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REMARKS

Claims 58-116 are now pending in the application. Claims 70-78, 85, 86, and 98-104 have been withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a non-elected invention. Claims 58-69, 79-84, 87-97, and 105-116 are being examined on the merits in the present paper. In addition, pursuant to the P.T.O.'s indication that claim 84 would be allowable if re-written in independent form, claim 84 is cancelled and the subject matter re-presented in independent form as claim 117. The P.T.O. is respectfully requested to reconsider and withdraw the rejections in view of the amendments and remarks contained herein.

Unity of Invention

Applicants acknowledge that the P.T.O. maintains its decision not to rejoin Groups I, II and IV. Applicants gratefully acknowledge the rejoining of co-examination of the claims of Group III with the claims of Group I. However, the meaning of the PTO's statement that that the invention of Group III is well known in the art and may be obvious to one of ordinary skill in the art is not clear to the Applicants. Applicants believe that the claims of Group III are novel and unobvious over the art.

Specification

The specification stands objected to for certain informalities. Applicants have amended the specification in accordance with the P.T.O.'s suggestions. Therefore, reconsideration and withdrawal of these objections are respectfully requested.

Claim Objections

The P.T.O. objected to certain claims as being inconsistent and/or grammatically incorrect. In the interest of rapid completion of prosecution of the present application, amendments based upon the P.T.O.'s suggestions have been adopted for claims 59, 60, 61, 80, 81, 82, 106, 107, and 108. No reduction in claim scope is intended by these amendments.

Rejections Under 35 U.S.C. § 112, Second Paragraph

Applicants request reconsideration of the rejection of Claims 61, 82, 90, and 105-116 rejected under 35 U.S.C. § 112, second paragraph because of the amendments to the claims. The P.T.O. indicated that it found the claim language unclear, and suggested changes. Amendments have been made to these claims in response to the PTO's suggestions. No reduction in claim scope is intended by these amendments. Applicants, therefore, request withdrawal of the rejection .

Rejections Under 35 U.S.C. § 112, Second Paragraph Regarding Written Description

Applicants request reconsideration of the rejection of Claims 58-69, 79-81, 83, 87-97, and 105-116 under 35 U.S.C. § 112, second paragraph regarding written description because a single representative species can suffice to describe adequately an entire genus under the standards set forth in *In re Marzocchi*, 169 U.S.P.Q. 367 (C.C.P.A. 1971), *Regents of the University of California v. Eli Lilly and Company*, 43 USPQ2d 1398 (Fed. Cir. 1997), and *Utter v. Hiraga*, 6 USPQ2d 1709 (Fed. Cir. 1988).

In *Marzocchi*, the C.C.P.A. held that written description under 35 U.S.C. § 112 first paragraph is presumed to be adequate, unless or until sufficient evidence or reasoning to the

contrary has been presented by the P.T.O. to rebut the presumption. 169 U.S.P.Q. at 370; M.P.E.P. § 2163.04. In *Regents v. Lilly*, the Federal Circuit stated that a "description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs ..." In *Regents*, the specification merely provided a single constructive example of a human cDNA. In that case, the Federal Circuit found the constructive example inadequate to show possession of the claimed invention (43 USPQ2d at 1404). The possibility of an actual example of a single species sufficing to represent a genus was not excluded by the Federal Circuit. By extension of the Federal Circuit's holding in *Regents*, description of a genus of microbes may be achieved if a representative number of species fall within the scope of the genus. In addition, in *Utter v. Hiraga*, 6 USPQ2d 1709, (Fed. Cir. 1988) the Federal Circuit stated that every species in a genus need not be described in order that a genus meet the written description requirement.

In the present application, the P.T.O.'s rejection rests on the assertion that only a single representative of species of each microbe is disclosed ("[a] single representative species of a first microbe (*E. coli* JWF1/pAD1.88A) or a second microbe (*G. oxydans* ATCC 621) is insufficient to provide a description of all species of first and second microbes...encompassed by the claims.") Firstly, the P.T.O.'s reasoning does not rebut the presumption of adequacy in this case, because the P.T.O. provides no reason why a disclosure of an actual representative species is inadequate to show possession of the invention. Secondly, examination of the NCBI nucleotide database website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=nucleotide) reveals 124 entries for nucleotide sequences encoding myo-inositol-1-phosphate synthase that antedate the priority date of the present application. These entries disclose nucleotide sequences encoding myo-inositol-1-phosphate synthase from species such as *Arabidopsis thaliana* (thale-cress), *Drosophila melanogaster* (fruit fly), *Homo sapiens* (human), *Hordeum vulgare* (barley),

Leishmania amazonensis (Leishmania), *Lycopersicon esculentum* (tomato), *Mesembryanthemum crystallinum* (common iceplant), *Mus musculus* (mouse), *Oryza sativa* (rice), *Rattus norvegicus* (rat), *Saccharomyces cerevisiae* (yeast), *Solanum sogarandinum* (potato), and *Zea mays* (corn). Therefore, at least as early as the time of filing of the priority application, one of ordinary skill in the art would appreciate that the inventors contemplated a variety of sources of nucleic acids encoding myo-inositol-1-phosphate synthase to construct a first microbe in addition to the disclosed examples. With regard to the second microbe, the specification recites, at least on page 5, lines 12-13, examples of two different microbial species that can be used for the second microbe (*Bacillus subtilis* and *Gluconobacter oxydans*). This disclosure of two exemplar species in the specification obviates the P.T.O.'s contention that "a single representative species of a... second microbe (*G. oxydans* ATCC 621) is insufficient to provide a description..." Furthermore, the specification states, on page 5, lines 10-12 that the second microbe can be "a naturally occurring microbe that express[es] inositol dehydrogenase activity." Examination of the NCBI nucleotide database website http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=nucleotide) reveals at least four entries antedating the priority date of the present application. These entries disclose microbes comprising inositol dehydrogenase (*Sinorhizobium meliloti, Saccharomyces cerevisiae, Mycobacterium leprae*, and *Bacillus subtilis*). These species provide examples of microbes expressing inositol dehydrogenase activity contemplated by the inventors. Because not all species in a genus need be described for a genus to meet the written description requirement (*Utter v. Hiraga*, 6 USPQ2d at 1714), Applicants request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph for written description.

Rejections Under 35 U.S.C. § 112, Second Paragraph Regarding Enablement

Applicants request reconsideration of the rejection of Claims 58-69, 79-81, 83, 87-97, and 105-116 under 35 U.S.C. § 112, second paragraph regarding enablement because the P.T.O. has not demonstrated that the claims are not enabled under the standards set forth in *In re Marzocchi,* 169 USPQ 367 (C.C.P.A. 1971), and *In re Wands*, 8 USPQ 2d 1400 (Fed. Cir. 1988). In *Marzocchi,* the Court of Customs and Patent Appeals stated that

> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. ... In any event, it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.

As will be explained below, the PTO has not met its burden of supporting its assertions with acceptable evidence or reasoning. The rejection will be addressed on the basis of the *Wands* factors cited by the PTO in the rejection.

Breadth of the claims. The PTO's rejection for enablement rests, in part, on the contention that the "extremely large number of microbes and conditions for acid catalyzed dehydration broadly encompassed by the claims" is not commensurate with the enablement provided by the disclosure. The PTO provides no support for this contention. Using claim 58 as an example, the PTO provides no reason why a person of ordinary skill in the art would not find the specification enabling for a first microbe comprising a recombinant DNA encoding *myo*-inositol phosphate synthase, a second microbe expressing inositol dehydrogenase activity, or

conversion of *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration. With regard to a microbe comprising a recombinant DNA encoding *myo*-inositol phosphate synthase, a search of the NCBI nucleotide database website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=nucleotide) for sequences of nucleic acids encoding this enzyme reveals 124 entries published prior to the priority date of the present application. These entries disclose nucleotide sequences encoding myo-inositol-1-phosphate synthase from species such as *Arabidopsis thaliana* (thale-cress), *Drosophila melanogaster* (fruit fly), *Homo sapiens* (human), *Hordeum vulgare* (barley), *Leishmania amazonensis* (Leishmania), *Lycopersicon esculentum* (tomato), *Mesembryanthemum crystallinum* (common iceplant), *Mus musculus* (mouse), *Oryza sativa* (rice), *Rattus norvegicus* (rat), *Saccharomyces cerevisiae* (yeast), *Solanum sogarandinum* (potato), and *Zea mays* (corn). Applicants contend that at the time of filing of the application, powerful technologies such as the polymerase chain reaction were available to workers in the art to obtain such nucleic acids. Therefore, using the available technologies, no more than routine experimentation by a person of ordinary skill in the art would be required to generate other microbes comprising a recombinant DNA encoding a *myo*-inositol-1-phosphate synthase. Similarly, with regard to a microbe expressing inositol dehydrogenase activity, a search of the NCBI nucleotide database website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=nucleotide) website reveals ten entries as of the priority date of the application for nucleic acids encoding inositol dehydrogenase. These entries disclose nucleic acid sequences encoding inositol dehydrogenase from microbial species such as *Sinorhizobium meliloti, Saccharomyces cerevisiae, Mycobacterium leprae*, and *Bacillus subtilis*. Applicants contend that as of the filing date, no more than routine experimentation by a person of ordinary skill in the art would be needed to

identify or select a microbe expressing inositol dehydrogenase activity. As for converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration, applicants contend that although the specification discloses the use of 0.5 M H_2SO_4, the PTO provides no reason why this conversion involves anything other than routine acid-catalyzed hydrolysis which can be achieved using a variety of acids, nor are any reasons provided demonstrating that determining the conditions for such hydrolysis under conditions different than those disclosed for 0.5 M H_2SO_4 would require undue experimentation. Applicants, therefore, request withdrawal of the rejection under 35 U.S.C. § 112 for enablement on the basis of breadth of the claims.

The amount of guidance and working examples. The PTO's rejection for enablement rests, in part, on the contention that the specification fails to provide additional guidance regarding other microorganisms or dehydration condition that my be successfully employed in the claimed methods. In particular, the PTO points to a teaching in the specification that conversion of *myo*-1-inositol-phosphate to *myo*-inositol is "fortuitously catalyzed in *E. coli* JWF1/pAD1.88A by unidentified cytosolic or periplasmic phosphatase activity." However, applicants note that phosphatases are virtually ubiquitous in cells. For example, the enzyme alkaline phosphatase is a phosphatase of broad substrate specificity that is present in the cytoplasm or periplasmic space of a large numbers of microorganisms. See, e.g., McComb et al., *Alkaline Phosphatase*, Plenum Press, New York, 1979, in particular pp. 30-31 and 868-869, photocopy enclosed. A search of the NCBI nucleotide database website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=nucleotide) website reveals 110 entries for sequences of alkaline phosphatase published prior to the filing date of the present enzyme. Most of the species sources listed are microbes. Furthermore, in addition to being nearly ubiquitous in distribution, alkaline phosphatase is also known to display phosphatase activity

against a wide range of substrates, for example, nucleic acids, proteins, small aliphatic compounds, (for example phosphate sugars), and aromatic compounds (for example *p*-nitrophenylphosphate) (*McComb et al., supra*). Indeed, alkaline phosphatase is known to hydrolyze phosphate from cyclohexanol phosphate, a compound closely related in structure to *myo*-1-inositol-phosphate (*McComb* at 306, photocopy enclosed). In addition, at least one other phosphatase, phytase (an acid phosphatase), is known to release phosphate from myo-inositol hexaphosphate, and is found in a wide variety of microbes (see, e.g., US Patent 6,451,572 to Lei, "Background of the Invention"; Russolini et al., *Cell Mol. Life Sci.* 54: 833-850, 1998 (enclosed)). Furthermore, phytases from *E. coli* are found in the periplasmic space, and can hydrolyze p-nitrophenyl phosphate (Greiner et al., *Arch. Biochem Biophys.* 303: 101-113, 1993, enclosed). It is, therefore, unsurprising that the conversion of *myo*-1-inositol-phosphate to *myo*-inositol is fortuitously catalyzed in *E. coli* JWF1/pAD1.88A by unidentified cytosolic or periplasmic phosphatase activity. Applicants, therefore, contend that because at least two phosphatases known to be capable of removing phosphates from compounds similar in structure *myo*-inositol phosphate are present in a wide variety of microbes including *E. coli*, the phosphatase activity catalyzing the removal of phosphate from *myo*-1-inositol-phosphate is unrelated to any biochemical activity specifically attributable to *E. coli* JWF1/pAD1.88A. Applicants, therefore, respectfully request withdrawal of the rejection under 35 U.S.C. § 112 for enablement on the basis of the P.T.O.'s contention that *E. coli* JWF1/pAD1.88A is required because of its unidentified phosphatase activity.

The PTO's rejection for enablement also rests, in part, on the contention that "the prior art teaches that the use of common acid catalysts gives rise to an aromatization of acyl derivatives of myo-2-inosose with formation of derivatives of 1,2,3,5-tetrahydroxybenze," citing

Posternak on page 164 for support. Applicants respectfully disagree with the PTO's interpretation of Posternak. Posternak states that "...the acyl derivatives of scylloinosose are prepared in the presence of acid catalysts, the use of basic catalysts giving rise to an aromatization with formation of derivatives of 1,2,3,5-tetrahydroxybenzene" [emphasis added]. Posternak is silent on whether acid catalysts give rise to aromatization of acyl derivatives of *myo*-2-inosose with formation of 1,2,3,5-tetrahydoxybenzene. Posternak is, therefore, inapposite to enablement of the present application.

Because the phosphatase activity catalyzing the removal of phosphate from *myo*-1-inositol-phosphate is unrelated to biochemical activity specifically attributable to *E. coli* JWF1/pAD1.88A, and because the teachings of Posternak are inapposite to the present application, applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112 for enablement on the basis of amount of guidance and working examples.

The unpredictability of the art. The PTO's rejection for enablement rests, in part, on the contention that "different microbes possess distinct metabolic pathways. Such pathways may not be optimized for the production of myo-inositol or myo-inosose and may instead produce some other product..." This reasoning presented is mere conjecture. To meet the standards of *In re Marzocchi*, however, the PTO must provide evidence that different microbes will (and not merely may) produce some other product instead of (and not merely in addition to) myo-inositol or myo-inosose. Because the PTO has not provided such evidence, applicants request withdrawal of the rejection under 35 U.S.C. § 112 for enablement on the basis of unpredictability of the art.

The quantity of experimentation necessary. The PTO's rejection for enablement rests, in part, on the contention that "it is not routine in the art to screen all microbes, particularly those having the required unidentified phosphatase activity, as encompassed by the claims to identify those microbes with a metabolic pathway amenable to the production of the intermediate product, *myo*-inositol and *myo*-2-inosose..." To meet the standards of *Marzocchi*, however, the PTO must provide evidence that different microbes are likely not to have the phosphatase activity. As discussed *supra*, however, microbes containing the appropriate enzyme activity are well known in the art to be widely distributed. Testing individual microbe species for the appropriate phosphatase activity is thus a matter of routine experimentation. Applicants, therefore, request withdrawal of the rejection under 35 U.S.C. § 112 for enablement on the basis of quantity of experimentation necessary.

In summary, the PTO has not met its burden of demonstrating that the Applicants have not provided sufficient guidance to enable one of skill in the art to make and use the claimed invention in a manner reasonably correlated with the scope of the claims. Applicants, therefore, respectfully request withdrawal of the rejection of claims 58-69, 79-81, 83, 87-97, and 105-116 under 35 U.S.C. § 112 for enablement.

Applicants request reconsideration and withdrawal of the rejection of Claims 61,63, 65, 84, 90, 92, 94, 108, 110 and 112 under 35 U.S.C. § 112, second paragraph regarding enablement because a declaration signed by a representative of the assignee of the application stating that the specific strains deposited in the American Type Culture Collection under the terms of the Budapest Treaty will irrevocably and without restriction or condition be released to the public upon the issuance of the patent is provided as an enclosure with this paper. This statement is supported by a copy of a certificate of deposit issued by the ATCC, also enclosed herein.

Allowable Subject Matter

The P.T.O. states that claim 84 would be allowable if rewritten in independent form. Accordingly, Applicants have cancelled claim 84 and added new claim 117 which includes the limitations of the base claim and any intervening claims. Therefore, claim 117 should be in condition for allowance.

Conclusion

It is believed that this amendment places the claims in a condition for allowance and such favorable action is respectfully requested. If, however, any of the claims are deemed by the P.T.O. not to be in a condition for allowance, Applicants request an interview with the P.T.O. so that any remaining issues can be resolved. Should any questions arise, the P.T.O. is requested to contact the undersigned attorney.

Respectfully submitted,

Dated: June 13, 2003

By: Saul L. Zackson

Saul L. Zackson
Reg. No. 52,391
Harness, Dickey & Pierce
7700 Bonhomme, Suite 400
St. Louis, Missouri 63105
(314) 726-7500

ALKALINE PHOSPHATASE

Robert B. McComb
George N. Bowers, Jr.

Hartford Hospital
Hartford, Connecticut

and

Solomon Posen

Sydney Hospital
Sydney, Australia

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3.4.2. Developmental Correlations

Slime molds exist as individual cells (designated amoebae) that generally feed on bacteria and undergo cell division.[35,36] When the local food supply is depleted, the cells aggregate and subsequently form a multicellular organized fruiting body (= sorocarp) supported by a stalk. Spores form and germinate into myxamoebae, and the cycle is repeated.

Alkaline phosphatase is found at all stages of the *D. discoideum* life cycle. Activity is highest in the tip of the young fruiting body and lowest in the spore mass.[35,36] High activities are also found at the tip of the developing stalk in such species as *D. mucoroides* and *D. purpureum*.[35] High concentrations of orthophosphate in the growth medium repress the synthesis of the enzyme.[38] Cycloheximide, an inhibitor of protein synthesis, suppresses the rise in alkaline phosphatase activity during sorocarp formation in *D. discoideium*.[39] Actinomycin D, an inhibitor of RNA synthesis, can also prevent this rise, provided it is added early during development.[39] Mutant strains that fail to aggregate or form sorocarps do not show the rise in alkaline phosphatase seen in the wild variety.[39]

3.4.3. Intracellular Localization and Solubilization

In amoebae of *D. discoideum*, alkaline phosphatase is tightly bound to the plasma membrane,[41,42] and detergents must be employed to achieve complete solubilization. Green and Newell,[41] who prepared plasma membrane subfractions, showed that one of these subfractions was selectively enriched for alkaline phosphatase, but not for other plasma-membrane-associated enzymes.

Parish and Pelli[42] presented evidence that the enzyme, which is associated with the cell surface, does not normally become internalized during phagocytosis. Colchicine alters this state of affairs; consequently, under its influence, intracellular membrane vacuoles show alkaline phosphatase activity.

3.5. Bacteria

3.5.1. Species

Large numbers of bacteria, particularly gram-negative organisms, have been known to synthesize alkaline phosphatase under suitable growth conditions (usually low phosphate content of the medium). Some of these bacteria are listed in Table 3.1.

3.5.2. Localization within Organisms

The enzyme is associated with the cell wall in *Eschericha coli*[43] and other gram-negative bacteria.[44] It is generally thought to be localized to the periplasmic space (Figure 3.2) between the cell wall and the cytoplasmic membrane.[43,45–50] Nisonson *et al.*[51] and Wetzel *et al.*,[48] who studied 5′nucleotidase and *E. coli* alkaline phosphatase, respectively, showed that the apparent distribution of the reaction products was strongly

TABLE 3.1. S

Species
Aerobacter aerogenes
Bacillus anthracis
B. cereus
B. licheniformis
B. megaterium
Bacillus spp.
B. subtilis
Bacteroides ruminicola
Escherichia coli
Halobacterium salinarium
Klebsiella aerogenes
Klebsiella spp.
Micrococcus sodonensis

influenced by the met
glutaraldehyde-fixed *E.*
while formalin fixation
monas aeruginosa, glu
surface.[52]

Wetzel *et al.*[48] sh
intense at the polar cap

Some bacteria, su
defective cell walls. Th
during growth. In son
bound to the cell-wall
of the cells.[55] Inouye
tem, using DNA or mes

FIGURE 3.2. Electron-mi
line phosphatase. The enz
arrows) and the plasma m
merization and zinc-bindin
have taken place (see Chap

TABLE 3.1. Some Bacteria Reported to Synthesize Alkaline Phosphatase

Species	Ref. no.	Species	Ref. no.
Aerobacter aerogenes	131	*Neisseria* spp.	152
Bacillus anthracis	132	*Proteus* spp.	150
B. cereus	133	*Pseudomonas aeuruginosa*	153
B. licheniformis	134	*Ps. fluorescens*	154
B. megaterium	133	*Pseudomonas* spp.	44
Bacillus spp.	53, 133, 135	*Salmonella typhimurium*	155, 156
B. subtilis	56, 137–142	*Serratia marcescens*	157, 158
Bacteroides ruminicola	55	*Serratia* spp.	150
Escherichia coli	57, 143–147	*Staphylococcus aureus*	143, 159–161
Halobacterium salinarium	148	*Thermus aquaticus*	136
Klebsiella aerogenes	149	*Thiobacillus thioparus*	1003
Klebsiella spp.	150	*Vibrio alginolyticus*	162
Micrococcus sodonensis	151		

influenced by the method of fixation and other cytochemical procedural details. Thus, glutaraldehyde-fixed *E. coli* organisms displayed lead precipitate in the periplasmic space, while formalin fixation caused the precipitate to appear on the cell surface.[48] In *Pseudomonas aeruginosa*, glutaraldehyde fixation caused the enzyme to appear on the cell surface.[52]

Wetzel *et al.*[48] showed that alkaline phosphatase activity in *E. coli* is particularly intense at the polar caps of these elongated organisms.

Some bacteria, such as certain mutants of *Bacillus subtilis*[53] and *E. coli*,[54] have defective cell walls. These organisms release alkaline phosphatase into the culture medium during growth. In some instances, as in *Bacteroides ruminicola*, the enzyme is firmly bound to the cell-wall structures and cannot be released except by complete disruption of the cells.[55] Inouye *et al.*[56] synthesized *E. coli* alkaline phosphatase in a cell-free system, using DNA or messenger RNA templates.

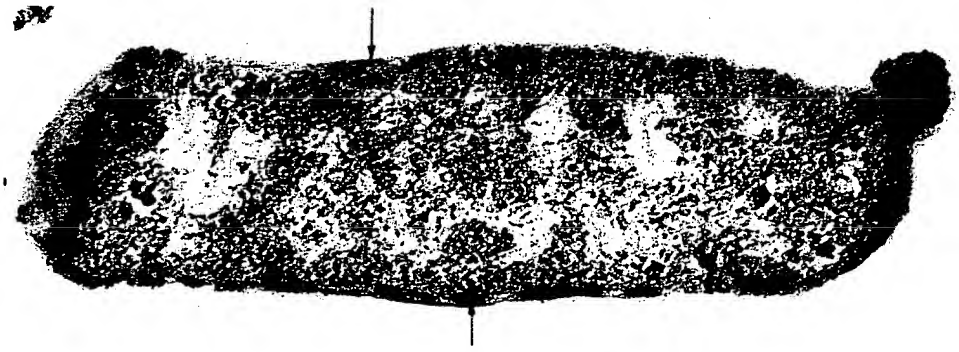

FIGURE 3.2. Electron-microscopic appearance of a single *Escherichia coli* organism stained for alkaline phosphatase. The enzyme is situated in the space between the outer membrane (indicated by arrows) and the plasma membrane. There is evidence that monomers migrate into this space, that dimerization and zinc-binding occur at this site, and that enzymatic activity is not seen until these events have taken place (see Chapter 5). From Done *et al.*[46] With permission.

TABLE 7.6. Selected References for Methods of Preparation of Substrates Employed in the Assay of Alkaline Phosphatases

Substrate	Author(s) and (year)	Ref. no.
p-Nitrophenylphosphate	Ohmori (1937)	7
	King and Nicholson (1939)	119
	Bessey and Love (1952)	120
	Aschaffenburg (1953)	121
Phenylphosphate	King and Nicholson (1939)	119
Cyclohexanol phosphate	King and Nicholson (1939)	119
p-Bromophenylphosphate	King and Nicholson (1939)	119
Phenolphthalein diphosphate	King (1943)	122
Phenol red diphosphate	King (1943)	122
Thymol blue diphosphate	King (1943)	122
Bromphenol blue diphosphate	King (1943)	122
Phenol tetrachlorphthalein diphosphate	King (1943)	122
Thymolphthalein diphosphate	King (1943)	122
Fluorescein phosphate	Neumann (1948)	123
o-Carboxyphenylphosphate	Hofstee (1954)	124
Naphthyl AS-MX phosphate[a]	Burstone (1958)	125
Naphthyl AS-BI phosphate[a]	Burstone (1958)	125
Naphthyl AS-BS phosphate	Burstone (1958)	125
Cysteamine-*S*-phosphate	Akerfeldt (1959)	126
Imidodiphosphate	Nielsen *et al.* (1961)	127
Tyrosine-*O*-phosphate	Mitchell and Lunan (1964)	128
Methylumbelliferylphosphate	Fernley and Walker (1965)	116
2,4-Dinitrophenylphosphate	Kirby and Varvoglis (1966)	129
Thymolphthalein monophosphate	Coleman (1966)	130
	Proksch (1972)	178
p-Toluidinium-5-bromo-6-chloro-3-indolyl phosphate	Horwitz *et al.* (1966)	131
5-Bromo-6-chloro-3-indolyl phosphate	Horwitz *et al.* (1966)	131
o-Nitrophenylphosphate[b]	Kirby and Varvoglis (1967)	132
p-Nitrophenylphosphorothioate	Breslow and Katz (1968)	133
Naphthol AS phosphate	Vaughan *et al.* (1971)	23
p-Phenylazophenylphosphate	Mushak and Coleman (1972)	134
N-(-α-naphthyl) phosphoramidate[b]	Snyder and Wilson (1972)	135
4-Formylphenylphosphate	Williams *et al.* (1973)	136
p-Phenylazophenyl-*O*-phosphorothioate	Chlebowski and Coleman (1974)	137

[a]See Figure 7.7. [b]Many other substrates also synthesized.

7.3.2. *Impurities*

Impurities may be present in substrates either because they were not removed at the time of synthesis or because of spontaneous hydrolysis during storage.

Salomon *et al.*[138] described the contamination of phenylphosphate by orthophosphate. Similar observations have been made in our laboratory. A preparation of disodium phenylphosphate, which had been stored for 10 years at room temperature, contained 10 mol% free phosphate but no measurable free phenol.[400] This suggests that the free phosphate was present from the time of synthesis and not as the result of spontaneous hydrolysis (also see Section 7.3.3).

12.2.3. Binding of Inorganic Phosphate

Alkaline phosphatases have a high affinity for inorganic orthophosphates, which bind competitively with phosphate esters (see Section 6.11.1.1). The binding of inorganic phosphate may cause conformational changes in the enzyme molecule (see Section 6.6.4), and the type of bonding varies with pH throughout the physiological range[111] (see Section 6.5.5). It is therefore possible that an enzyme-phosphate complex oscillates rapidly between the intra- and extracellular media, and that phosphatase action *in vivo* relates to phosphate transport.

However, the ability of alkaline phosphatase to ligate with orthophosphate *in vitro* does not prove any sort of physiological function,[18] nor does it imply that alkaline phosphatase is the sole or even the primary phosphate-transport protein. Other phosphate-binding proteins have been identified in the periplasmic space of *E. coli*,[19,20] and while these proteins share some genetic regulatory mechanisms with alkaline phosphatase,[21] they are structurally different.[21-24]

The possible relationship between alkaline phosphatase and phosphate transport is further discussed in Section 12.5.5.

12.3. Distribution in Nature as a Clue to Function

> Where a substance is being moved in considerable quantity across a cell membrane by an active process, then there is very likely to be present in the cell membrane a high concentration of alkaline phosphatase.[25]

12.3.1. Distribution in Microorganisms

In microorganisms, alkaline phosphatase is often located in the periplasmic space (the area between the cell wall and the cell membrane).[26,27] Malamy and Horecker[26] speculated that the localization of the enzyme outside the cytoplasm provides it with ready access to substrates in the culture medium, while its absence from the interior of the cell allows the organism to conserve its pool of phosphate esters. Malamy and Horecker[26] showed that *E. coli* organisms rapidly hydrolyze ^{32}P-labeled glucose-6-phosphate in the medium and retain approximately 30% of the liberated labeled phosphate in the cell even in the presence of excess inorganic phosphate. Malamy and Horecker[26] concluded that the organisms take up labeled phosphate (originating from glucose-6-phosphate) preferentially, and that alkaline phosphatase is responsible for the hydrolysis of this substrate (also see Section 12.6.2).

Schlesinger[28] showed that the active dimeric form of *E. coli* alkaline phosphatase is assembled in the periplasmic space, and speculated that this arrangement protects the bacterial cell from the presence of an active alkaline phosphatase in its cytoplasm. Tribhuwan and Pradhan[267] showed that substances such as procaine hydrochloride that alter the structure of cell membranes diminish the formation of *E. coli* alkaline phosphatase dimers. Monomer formation and the synthesis of intracellular enzymes are not affected by these agents.[267]

Inouye and Beckwith[270] have presented evidence that the first product in the synthesis of *E. coli* alkaline phosphatase by an *in vitro* system[272] is several thousand daltons heavier than the natural *E. coli* monomer. The precursor could be converted to a fragment similar to the subunit of native *E. coli* alkaline phosphatase by an outer-membrane fraction of the *E. coli* cell. The precursor is more hydrophobic than the native subunit,[269] which suggests a mechanism for passage of this protein material from its site of synthesis[271] through the highly hydrophobic layer of the bacterial inner membrane to its final location in the periplasmic space.

12.3.2. Distribution in Plants

Most plants are devoid of alkaline phosphatase (see Chapter 3, Section 3.2). If the hydrolysis of phosphate esters constitutes the principal "function" of alkaline phosphatases, then presumably plants either have no "need" of such enzymes or this function is fullfilled by alternate "phosphatases."

Some parasitic plants are said to show alkaline phosphatase activity in tissues that interface with the host plant.[29,30]

12.3.3. Distribution in Parasites

The integument of higher animals usually contains little, if any, alkaline phosphatase (see Section 3.19.1). Cestodes, which absorb most of their nutrients through their cuticles, have their alkaline phosphatase localized almost exclusively in this structure[31–33] (see Section 3.9.3). Adult tapeworms, which absorb nutrients from the medium more efficiently than larval forms,[34] also have greater alkaline phosphatase activities.[31,32]

In adult *Schistosoma mansoni*, alkaline phosphatase is found in the cuticle,[33] with different distribution in males and females. Sex-related differences also exist in this species with regard to the host-parasite interface,[35] with alkaline phosphatase activity localized in that portion of the cuticle that is in contact with host tissues (see Section 3.9.2.2).

12.3.4. Absence from Aschelminthes (Nematodes)

Many species in this phylum are devoid of alkaline phosphatase (see Section 3.11). In contrast, the host reaction to these parasites includes high concentrations of the enzyme in areas surrounding the parasite.[36–38] The phosphatase-free worms are killed by levamisole which inhibits a number of alkaline phosphatases[39] (see Sections 6.11.2.5, 7.7.3.3, and 8.2.5.7.7), including the host enzyme surrounding the parasites.[38] The relationship between the antihelminthic action of levamisole and its role as an alkaline phosphatase inhibitor is not understood.

12.3.5. Distribution in Digestive Systems

Specialized absorptive cells almost invariably show high alkaline phosphatase activity, though there are notable exceptions (see Section 12.3.4). In a number of invertebrate species,[40] food ingestion is associated with a demonstrable rise in intestinal alkaline phospha-

FULL TEXT OF CASES (USPQ2D)
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Decided September 30, 1988
No. 87-1454

Headnotes

PATENTS

1. Patentability/Validity -- Adequacy of disclosure (§ 115.12)

Data disclosed in application for immunoassay method patent, which shows that applicants screened nine of 143 cell lines developed for production of antibody necessary to practice invention, stored remainder of said cell lines, and found that four out of nine cell lines screened produced antibody falling within limitation of claims, were erroneously interpreted by Board of Patent Appeals and Interferences as failing to meet disclosure requirements of 35 USC 112, since board's characterization of stored cell lines as "failures" demonstrating unreliability of applicants' methods was improper in view of fact that such unscreened cell lines prove nothing concerning probability of success of person skilled in art attempting to obtain requisite antibodies using applicants' methods.

2. Patentability/Validity -- Adequacy of disclosure (§ 115.12)

Disclosure in application for immunoassay method patent does not fail to meet enablement requirement of 35 USC 112 by requiring "undue experimentation," even though production of monoclonal antibodies necessary to practice invention first requires production and screening of numerous antibody producing cells or "hybridomas," since practitioners of art are prepared to screen negative hybridomas in order to find those that produce desired antibodies, since in monoclonal antibody art one "experiment" is not simply screening of one hybridoma but rather is entire attempt to make desired antibody, and since record indicates that amount of effort needed to obtain desired antibodies is not excessive, in view of applicants' success in each attempt to produce antibody that satisfied all claim limitations.

Case History and Disposition:

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Appeal from decision of Patent and Trademark Office, Board of Patent Appeals and Interferences.

Application for patent of Jack R. Wands, Vincent R. Zurawski, Jr., and Hubert J. P. Schoemaker, serial number 188,735. From decision of Board of Patent Appeals and Interferences affirming rejection of application, applicants appeal. Reversed; Newman, J., concurring in part and dissenting in part in separate opinion.

Attorneys:
Jorge A. Goldstein, of Saidman, Sterne, Kessler & Goldstein (Henry N. Wixon, with them on brief), Washington, D.C., for appellant.

John H. Raubitschek, associate solicitor (Joseph F. Nakamura and Fred E. McKelvey, with him on brief), PTO, for appellee.

Judge:
Before Smith, Newman, and Bissell, circuit judges.

Opinion Text

Opinion By:
Smith, J.

This appeal is from the decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (board) affirming the rejection of all remaining claims in appellant's application for a patent, serial No. 188,735, entitled "Immunoassay Utilizing Monoclonal High Affinity IgM

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Antibodies," which was filed September 19, 1980. 1 The rejection under 35 U.S.C. §112, first paragraph, is based on the grounds that appellant's written specification would not enable a person skilled in the art to make the monoclonal antibodies that are needed to practice the claimed invention without undue experimentation. We reverse.

I. *Issue*

The only issue on appeal is whether the board erred, as a matter of law, by sustaining the examiner's rejection for lack of enablement under 35 U.S.C. §112, first paragraph, of all remaining claims in appellants' patent application, serial No. 188,735.

II. *Background*

A. *The Art*.

The claimed invention involves immunoassay methods for the detection of hepatitis B surface antigen

by using high-affinity monoclonal antibodies of the IgM isotype. *Antibodies* are a class of proteins (immunoglobulins) that help defend the body against invaders such as viruses and bacteria. An antibody has the potential to bind tightly to another molecule, which molecule is called an antigen. The body has the ability to make millions of different antibodies that bind to different antigens. However, it is only after exposure of an antigen that a complicated *immune response* leads to the production of antibodies against that antigen. For example, on the surface of hepatitis B virus particles there is a large protein called *hepatitis B surface antigen* (HBsAg). As its name implies, it is capable of serving as an antigen. During a hepatitis B infection (or when purified HBsAg is injected experimentally), the body begins to make antibodies that bind tightly and specifically to HBsAg. Such antibodies can be used as reagents for sensitive diagnostic tests (*e.g* ., to detect hepatitis B virus in blood and other tissues, a purpose of the claimed invention). A method for detecting or measuring antigens by using antibodies as reagents is called an *immunoassay* .

Normally, many different antibodies are produced against each antigen. One reason for this diversity is that different antibodies are produced that bind to different regions (determinants) of a large antigen molecule such as HBsAg. In addition, different antibodies may be produced that bind to the same determinant. These usually differ in the tightness with which they bind to the determinant. *Affinity* is a quantitative measure of the strength of antibody-antigen binding. Usually an antibody with a higher affinity for an antigen will be more useful for immunological diagnostic tests than one with a lower affinity. Another source of heterogeneity is that there are several immunoglobulin classes or *isotypes* . Immunoglobulin G (IgG) is the most common isotype in serum. Another isotype, immunoglobulin M (IgM), is prominent early in the immune response. IgM molecules are larger than IgG molecules, and have 10 antigen-binding sites instead of the 2 that are present in IgG. Most immunoassay methods use IgG, but the claimed invention uses only IgM antibodies.

For commercial applications there are many disadvantages to using antibodies from serum. Serum contains a complex mixture of antibodies against the antigen of interest within a much larger pool of antibodies directed at other antigens. There are available only in a limited supply that ends when the donor dies. The goal of monoclonal antibody technology is to produce an unlimited supply of a single purified antibody.

The blood cells that make antibodies are *lymphocytes* . Each lymphocyte makes only one kind of antibody. During an immune response, lymphocytes exposed to their particular antigen divide and mature. Each produces a *clone* of identical daughter cells, all of which secrete the same antibody. Clones of lymphocytes, all derived from a single lymphocyte, could provide a source of a single homogeneous antibody. However, lymphocytes do not survive for long outside of the body in cell culture.

Hybridoma technology provides a way to obtain large numbers of cells that all produce the same antibody. This method takes advantage of the properties of *myeloma* cells derived from a tumor of the immune system. The cancerous myeloma cells can divide indefinitely in vitro. They also have the potential ability to secrete antibodies. By appropriate experimental manipulations, a myeloma cell can be made to fuse with a lymphocyte to produce a single hybrid cell (hence, a hybridoma) that contains the genetic material of both cells. The hybridoma secretes the same antibody that was made by its parent lymphocyte, but acquires the capability of the myeloma cell to divide and grow indefinitely in cell culture. Antibodies produced by a clone of hybridoma cells (i.e., by hybridoma

cells that are all progeny of a single cell) are called monoclonal antibodies. 2

B. *The Claimed Invention* .

The claimed invention involves methods for the immunoassay of HBsAg by using high-affinity monoclonal IgM antibodies. Jack R. Wands and Vincent R. Zurawski, Jr., two of the three coinventors of the present application, disclosed methods for producing monoclonal antibodies against HBsAg in

United States patent No. 4,271,145 (the '145 patent), entitled "Process for Producing Antibodies to Hepatitis Virus and Cell Lines Therefor," which patent issued on June 2, 1981. The '145 patent is incorporated by reference into the application on appeal. The specification of the '145 patent teaches a procedure for immunizing mice against HBsAg, and the use of lymphocytes from these mice to produce hybridomas that secrete monoclonal antibodies specific for HBsAg. The '145 patent discloses that this procedure yields both IgG and IgM antibodies with high-affinity binding to HBsAg. For the stated purpose of complying with the best mode requirement of 35 U.S.C. §112, first paragraph, a hybridoma cell line that secretes IgM antibodies against HBsAg (the 1F8 cell line) was deposited at the American Type Culture Collection, a recognized cell depository, and became available to the public when the '145 patent issued.

The application on appeal claims methods for immunoassay of HBsAg using monoclonal antibodies such as those described in the '145 patent. Most immunoassay methods have used monoclonal antibodies of the IgG isotype. IgM antibodies were disfavored in the prior art because of their sensitivity to reducing agents and their tendency to self-aggregate and precipitate. Appellants found that their monoclonal IgM antibodies could be used for immunoassay of HbsAg with unexpectedly high sensitivity and specificity. Claims 1, 3, 7, 8, 14, and 15 are drawn to methods for the immunoassay of HBsAg using high-affinity IgM monoclonal antibodies. Claims 19 and 25-27 are for chemically modified (*e.g* ., radioactively labeled) monoclonal IgM antibodies used in the assays. The broadest method claim reads:

1. An immunoassay method utilizing an antibody to assay for a substance comprising hepatitis B-surface antigen (HBsAg) determinants which comprises the steps of:

contacting a test sample containing said substance comprising HBsAg determinants with said antibody; and

determining the presence of said substance in said sample;

wherein said antibody is a monoclonal high affinity IgM antibody having a binding affinity constant for said HBsAg determinants of at least $10^{9}M^{-1}$.

Certain claims were rejected under 35 U.S.C. §103; these rejections have not been appealed. Remaining claims 1, 3, 7, 8, 14, 15, 19, and 25-27 were rejected under 35 U.S.C. §112, first paragraph, on the grounds that the disclosure would not enable a person skilled in the art to make and use the invention without undue experimentation. The rejection is directed solely to whether the specification enables one skilled in the art to make the monoclonal antibodies that are needed to practice the invention. The position of the PTO is that data presented by Wands show that the production of high-affinity IgM anti-HBsAg antibodies is unpredictable and unreliable, so that it would require undue experimentation for one skilled in the art to make the antibodies.

III. *Analysis*

A. *Enablement by Deposit of Micro-organisms and Cell Lines* .

The first paragraph of 35 U.S.C. §112 requires that the specification of a patent must enable a person skilled in the art to make and use the claimed invention. "Patents * * * are written to enable those skilled in the art to practice the invention." 3 A patent need not disclose what is well known in the art. 4 Although we review underlying facts found by the board under a "clearly erroneous" standard, 5 we review enablement as a question of law. 6

Where an invention depends on the use of living materials such as microorganisms or

cultured cells, it may be impossible to enable the public to make the invention (i.e., to obtain these living materials) solely by means of a written disclosure. One means that has been developed for complying with the enablement requirement is to deposit the living materials in cell depositories which will distribute samples to the public who wish to practice the invention after the patent issues. 7 Administrative guidelines and judicial decisions have clarified the conditions under which a deposit of

organisms can satisfy the requirements of section 112. 8 A deposit has been held necessary for enablement where the starting materials (i.e., the living cells used to practice the invention, or cells from which the required cells can be produced) are not readily available to the public. 9 Even when starting materials are available, a deposit has been necessary where it would require undue experimentation to make the cells of the invention from the starting materials. 10

In addition to satisfying the enablement requirement, deposit of organisms also can be used to establish the filing date of the application as the prima facie date of invention, 11 and to satisfy the requirement under 35 U.S.C. §114 that the PTO be guaranteed access to the invention during pendency of the application. 12 Although a deposit may serve these purposes, we recognized, in *In re Lundak* , 13 that these purposes, nevertheless, may be met in ways other than by making a deposit.

A deposit also may satisfy the best mode requirement of section 112, first paragraph, and it is for this reason that the 1F8 hybridoma was deposited in connection with the '145 patent and the current application. Wands does not challenge the statements by the examiner to the effect that, although the deposited 1F8 line enables the public to perform immunoassays with antibodies produced by that single hybridoma, the deposit does not enable the generic claims that are on appeal. The examiner rejected the claims on the grounds that the written disclosure was not enabling and that the deposit was inadequate. Since we hold that the written disclosure fully enables the claimed invention, we need not reach the question of the adequacy of deposits.

B. *Undue Experimentation* .

Although inventions involving microorganisms or other living cells often can be enabled by a deposit, 14 a deposit is not always necessary to satisfy the enablement requirement. 15 No deposit is necessary if the biological organisms can be obtained from readily available sources or derived from readily available starting materials through routine screening that does not require undue experimentation. 16 Whether the specification in an application involving living cells (here, hybridomas) is enabled without a deposit must be decided on the facts of the particular case. 17

Appellants contend that their written specification fully enables the practice of

their claimed invention because the monoclonal antibodies needed to perform the immunoassays can be made from readily available starting materials using methods that are well known in the monoclonal antibody art. Wands states that application of these methods to make high-affinity IgM anti-HBsAg antibodies requires only routine screening, and that does not amount to undue experimentation. There is no challenge to their contention that the starting materials (i.e., mice, HBsAg antigen, and myeloma cells) are available to the public. The PTO concedes that the methods used to prepare hybridomas and to screen them for high-affinity IgM antibodies against HBsAg were either well known in the monoclonal antibody art or adequately disclosed in the '145 patent and in the current application. This is consistent with this court's recognition with respect to another patent application that methods for obtaining and screening monoclonal antibodies were well known in 1980. 18 The sole issue is whether, in this particular case, it would require undue experimentation to produce high-affinity IgM monoclonal antibodies.

Enablement is not precluded by the necessity for some experimentation such as routine screening. 19 However, experimentation needed to practice the invention must not be undue experimentation. 20 "the key word is 'undue,' not 'experimentation.' " 21

The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. Ansul Co. v. Uniroyal, Inc. [448 F.2d 872, 878-79; 169 USPQ 759, 762-63 (2d Cir. 1971), *cert. denied* , 404 U.S. 1018 [172 USPQ 257] (1972)]. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation

should proceed * * * . 22
The term "undue experimentation" does not appear in the statute, but it is well established that enablement requires that the specification teach those in the art to make and use the invention without undue experimentation. 23 Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing many factual considerations. The board concluded that undue experimentation would be needed to practice the invention on the basis of experimental data presented by Wands. These data are not in dispute. However, Wands and the board disagree strongly on the conclusion that should be drawn from that data.
Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman* . 24 They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. 25
In order to understand whether the rejection was proper, it is necessary to discuss further the methods for making specific monoclonal antibodies. The first step for making monoclonal antibodies is to immunize an animal. The '145 patent provides a detailed description of procedures for immunizing a specific strain of mice against HBsAg. Next the spleen, an organ rich in lymphocytes, is removed and the lymphocytes are separated from the other spleen cells. The lymphocytes are mixed with myeloma cells, and the mixture is treated to cause a few of the cells to fuse with each other. Hybridoma cells that secrete the desired antibodies then must be isolated from the enormous number of other cells in the mixture. This is done through a series of screening procedures.
The first step is to separate the hybridoma cells from unfused lymphocytes and myeloma cells. The cells are cultured in a medi

um in which all the lymphocytes and myeloma cells die, and only the hybridoma cells survive. The next step is to isolate and clone hybridomas that make antibodies that bind to the antigen of interest. Single hybridoma cells are placed in separate chambers and are allowed to grow and divide. After there are enough cells in the clone to produce sufficient quantities of antibody to analyze, the antibody is assayed to determine whether it binds to the antigen. Generally, antibodies from many clones do not bind the antigen, and these clones are discarded. However, by screening enough clones (often hundreds at a time), hybridomas may be found that secrete antibodies against the antigen of interest.
Wands used a commercially available radioimmunoassay kit to screen clones for cells that produce antibodies directed against HBsAg. In this assay the amount of radioactivity bound gives some indication of the strength of the antibody-antigen binding, but does not yield a numerical affinity constant, which must be measured using the more laborious Scatchard analysis. In order to determine which anti-HBsAg antibodies satisfy all of the limitations of appellants' claims, the antibodies require further screening to select those which have an IgM isotype and have a binding affinity constant of at least $10^{9}M^{-1}$. 26 The PTO does not question that the screening techniques used by Wands were well known in the monoclonal antibody art.
During prosecution Wands submitted a declaration under 37 C.F.R §1.132 providing information about all of the hybridomas that appellants had produced before filing the patent application. The first four fusions were unsuccessful and produced no hybridomas. The next six fusion experiments all produced hybridomas that made antibodies specific for HBsAg. Antibodies that bound at least 10,000 cpm in the commercial radioimmunoassay were classified as "high binders." Using this criterion, 143 high-binding hybridomas were obtained. In the declaration, Wands stated that 27
It is generally accepted in the art that, among those antibodies which are binders with 50,000 cpm or higher, there is a very high likelihood that high affinity (Ka [greater than] $10^{9}M^{-1}$) antibodies will be found. However, high affinity antibodies can also be found among high binders of between 10,000 and 50,000, as is clearly demonstrated in the Table.

The PTO has not challenged this statement.
The declaration stated that a few of the high-binding monoclonal antibodies from two fusions were chosen for further screening. The remainder of the antibodies and the hybridomas that produced them were saved by freezing. Only nine antibodies were subjected to further analysis. Four (three from one fusion and one from another fusion) fell within the claims, that is, were IgM antibodies and had a binding affinity constant of at least $10^{9}M^{-1}$. Of the remaining five antibodies, three were found to be IgG, while the other two were IgM for which the affinity constants were not measured (although both showed binding well above 50,000 cpm).
Apparently none of the frozen cell lines received any further analysis. The declaration explains that after useful high-affinity IgM monoclonal antibodies to HBsAg had been found, it was considered unnecessary to return to the stored antibodies to screen for more IgMs. Wands says that the existence of the stored hybridomas was disclosed to the PTO to comply with the requirement under 37 C.F.R. §1.56 that applicants fully disclose all of their relevant data, and not just favorable results. 28 How these stored hybridomas are viewed is central to the positions of the parties.
The position of the board emphasizes the fact that since the stored cell lines were not completely tested, there is no proof that any of them are IgM antibodies with a binding affinity constant of at least $10^{9}M^{-1}$. Thus, only 4 out of 143 hybridomas, or 2.8 percent, were *proved* to fall within the claims. Furthermore, antibodies that were proved to be high-affinity IgM came from only 2 of 10 fusion experiments. These statistics are viewed by the board as evidence that appellants' methods were not predictable or reproducible. The board concludes that Wands' low rate of demonstrated success shows that a person skilled in the art would have to

engage in undue experimentation in order to make antibodies that fall within the claims.
Wands views the data quite differently. Only nine hybridomas were actually analyzed beyond the initial screening for HBsAg binding. Of these, four produced antibodies that fell within the claims, a respectable 44 percent rate of success. (Furthermore, since the two additional IgM antibodies for which the affinity constants were never measured showed binding in excess of 50,000 cpm, it is likely that these also fall within the claims.) Wands argues that the remaining 134 unanalyzed, stored cell lines should not be written off as failures. Instead, if anything, they represent partial success. Each of the stored hybridomas had been shown to produce a high-binding antibody specific for HBsAg. Many of these antibodies showed binding above 50,000 cpm and are thus highly likely to have a binding affinity constant of at least $10^{9}M^{-1}$. Extrapolating from the nine hybridomas that were screened for isotype (and from what is well known in the monoclonal antibody art about isotype frequency), it is reasonable to assume that the stored cells include some that produce IgM. Thus, if the 134 incompletely analyzed cell lines are considered at all, they provide some support (albeit without rigorous proof) to the view that hybridomas falling within the claims are not so rare that undue experimentation would be needed to make them.
The first four fusion attempts were failures, while high-binding antibodies were produced in the next six fusions. Appellants contend that the initial failures occurred because they had not yet learned to fuse cells successfully. Once they became skilled in the art, they invariably obtained numerous hybridomas that made high-binding antibodies against HBsAg and, in each fusion where they determined isotype and binding affinity they obtained hybridomas that fell within the claims.
Wands also submitted a second declaration under 37 C.F.R. §1.132 stating that after the patent application was submitted they performed an eleventh fusion experiment and obtained another hybridoma that made a high-affinity IgM anti-HBsAg antibody. No information was provided about the number of clones screened in that experiment. The board determined that, because there was no indication as to the number of hybridomas screened, this declaration had very little value. While we agree that it would have been preferable if Wands had included this information, the declaration does show that when appellants repeated their procedures they again obtained a hybridoma that produced an

antibody that fit all of the limitations of their claims.
[1] We conclude that the board's interpretation of the data is erroneous. It is strained and unduly harsh to classify the stored cell lines (each of which was proved to make high-binding antibodies against HBsAg) as failures demonstrating that Wands' methods are unpredictable or unreliable. 29 At worst, they prove nothing at all about the probability of success, and merely show that appellants were prudent in not discarding cells that might someday prove useful. At best, they show that high-binding antibodies, the starting materials for IgM screening and Scatchard analysis, can be produced in large numbers. The PTO's position leads to the absurd conclusion that the more hybridomas an applicant makes and saves without testing the less predictable the applicant's results become. Furthermore, Wands' explanation that the first four attempts at cell fusion failed only because they had not yet learned to perform fusions properly is reasonable in view of the fact that the next six fusions were all successful. The record indicates that cell fusion is a technique that is well known to those of ordinary skill in the monoclonal antibody art, and there has been no claim that the fusion step should be more difficult or unreliable where the antigen is HBsAg than it would be for other antigens.
[2] When Wands' data is interpreted in a reasonable manner, analysis considering the factors enumerated in *Ex parte Forman* leads to the conclusion that undue experimentation would not be required to practice the invention. Wands' disclosure provides considerable direction and guidance on how to practice their invention and presents working examples. There was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known.
The nature of monoclonal antibody technology is that it involves screening hybridomas to determine which ones secrete antibody with desired characteristics. Practitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody. No evidence was presented by either party on how many hybridomas would be viewed by those in the art as requiring undue experimentation to screen. However, it seems unlikely that un

due experimentation would be defined in terms of the number of hybridomas that were never screened. Furthermore, in the monoclonal antibody art it appears that an "experiment" is not simply the screening of a single hybridoma, but is rather the entire attempt to make a monoclonal antibody against a particular antigen. This process entails immunizing animals, fusing lymphocytes from the immunized animals with myeloma cells to make hybridomas, cloning the hybridomas, and screening the antibodies produced by the hybridomas for the desired characteristics. Wands carried out this entire procedure three times, and was successful each time in making at least one antibody that satisfied all of the claim limitations. Reasonably interpreted, Wands' record indicates that, in the production of high-affinity IgM antibodies against HBsAG, the amount of effort needed to obtain such antibodies is not excessive. Wands' evidence thus effectively rebuts the examiner's challenge to the enablement of their disclosure. 30

IV. *Conclusion*

Considering all of the factors, we conclude that it would not require undue experimentation to obtain antibodies needed to practice the claimed invention. Accordingly, the rejection of Wands' claims for lack of enablement under 35 U.S.C. §112, first paragraph, is reversed.

REVERSED

Footnotes

Footnote 1. *In re Wands* , Appeal No. 673-76 (Bd. Pat. App. & Int. Dec. 30, 1986).
Footnote 2. For a concise description of monoclonal antibodies and their use in immunoassay see

Hybritech, Inc. v. Monoclonal Antibodies, Inc ., 802 F.2d 1367, 1368-71, 231 USPQ 81, 82-83 (Fed. Cir. 1986), *cert. denied* , 107 S.Ct. 1606 (1987).
Footnote 3. *W.L. Gore & Assocs., Inc. v. Garlock, Inc* ., 721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983), *cert. denied* , 469 U.S. 851 (1984).
Footnote 4. *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co* ., 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).
Footnote 5. *Coleman v. Dines* , 754 F.2d 353, 356, 224 USPQ 857, 859 (Fed. Cir. 1985).
Footnote 6. *Moleculon Research Corp. v. CBS, Inc* ., 793 F.2d 1261, 1268, 229 USPQ 805, 810 (Fed. Cir. 1986), *cert. denied* , 107 S.Ct. 875 (1987); *Raytheon Co. v. Roper Corp* ., 724 F.2d 951, 960 n.6, 220 USPQ 592 , 599 n.6 (Fed. Cir. 1983), *cert. denied* , 469 U.S. 835 [225 USPQ 232] (1984).
Footnote 7. *In re Argoudelis* , 434 F.2d 1390, 1392-93, 168 USPQ 99, 101-02 (CCPA 1970).
Footnote 8. *In re Lundak* , 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985); *Feldman v. Aunstrup* , 517 F.2d 1351, 186 USPQ 108 (CCPA 1975), *cert. denied* , 424 U.S. 912 [188 USPQ 720] (1976); Manual of Patent Examining Procedure (MPEP) 608.01 (p)(C) (5th ed. 1983, rev. 1987). *See generally* Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement* , 67 J. Pat. Trademark Off. Soc'y 569 (1985).
Footnote 9. *In re Jackson* , 217 USPQ 804, 807-08 (Bd. App. 1982) (strains of a newly discovered species of bacteria isolated from nature); *Feldman* , 517 F.2d 1351, 186 USPQ 108 (uncommon fungus isolated from nature); *In re Argoudelis* , 434 F.2d at 1392, 168 USPQ at 102 (novel strain of antibiotic-producing microorganism isolated from nature); *In re Kropp* , 143 USPQ 148, 152 (Bd. App. 1959) (newly discovered microorganism isolated from soil).
Footnote 10. *Ex parte Forman* , 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986) (genetically engineered bacteria where the specification provided insufficient information about the amount of time and effort required); *In re Lundak* , 773 F.2d 1216, 227 USPQ 90 (unique cell line produced from another cell line by mutagenesis).
Footnote 11. *In re Lundak* , 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman* , 517 F.2d at 1355, 186 USPQ at 113; *In re Argoudelis* , 434 F.2d at 1394-96, 168 USPQ at 103-04 (Baldwin, J. concurring).
Footnote 12. *In re Lundak* , 773 F.2d at 1222, 227 USPQ at 95-96; *In re Feldman* , 517 F.2d at 1354, 186 USPQ at 112.
Footnote 13. *In re Lundak* , 773 F.2d at 1222, 227 USPQ at 95-96.
Footnote 14. *In re Argoudelis* , 434 F.2d at 1393, 168 USPQ at 102.
Footnote 15. *Tabuchi v. Nubel* , 559 F.2d 1183, 194 USPQ 521 (CCPA 1977).
Footnote 16. *Id* . at 1186-87, 194 USPQ at 525; *Merck & Co. v. Chase Chem. Co* ., 273 F.Supp. 68, 77, 155 USPQ 139, 146 (D.N.J. 1967); *Guaranty Trust Co. v. Union Solvents Corp* ., 54 F.2d 400, 403-06, 12 USPQ 47, 50-53 (D. Del. 1931), *aff'd* , 61 F.2d 1041, 15 USPQ 237 (3d Cir. 1932), *cert. denied* , 288 U.S. 614 (1933); MPEP 608.01 (p)(C) ("No problem exists when the microorganisms used are known and readily available to the public.").
Footnote 17. *In re Jackson* , 217 USPQ at 807; *see In re Metcalfe* , 410 F.2d 1378, 1382, 161 USPQ 789, 792 (CCPA 1969).
Footnote 18. *Hybritech* , 802 F.2d at 1384, 231 USPQ at 94.
Footnote 19. *Id.; Atlas Powder Co. v. E.I. DuPont De Nemours & Co* ., 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984); *In re Angstadt* , 537 F.2d at 502-504, 190 USPQ at 218; *In re Geerdes* , 491 F.2d 1260, 1265, 180 USPQ 789, 793 (CCPA 1974); *Mineral Separation, Ltd. v. Hyde* , 242 U.S. 261, 270-71 (1916).
Footnote 20. *Hybritech* , 802 F.2d at 1384, 231 USPQ at 94; *W.L. Gore* , 721 F.2d at 1557, 220 USPQ at 316; *In re Colianni* , 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977) (Miller, J., concurring).
Footnote 21. *In re Angstadt* , 537 F.2d at 504, 190 USPQ at 219.
Footnote 22. *In re Jackson* , 217 USPQ at 807.
Footnote 23. *See Hybritech* , 802 F.2d at 1384, 231 USPQ at 94; *Atlas Powder* , 750 F.2d at 1576, 224 USPQ at 413.

Footnote 24. *Ex parte Forman* , 230 USPQ at 547.
Footnote 25. *Id.; see In re Colianni* , 561 F.2d at 224, 195 USPQ at 153 (Miller, J., concurring); *In re Rainer* , 347 F.2d 574, 577, 146 USPQ 218, 221 (CCPA 1965).
Footnote 26. The examiner, the board, and Wands all point out that, technically, the strength of antibody-HBsAg binding is measured as *avidity* , which takes into account multiple determinants on the HBsAg molecule, rather than affinity. Nevertheless, despite this correction, all parties then continued to use the term "affinity." We will use the terminology of the parties. Following the usage of the parties, we will also use the term "high-affinity" as essentially synonymous with "having a binding affinity constant of at least $10^{9}M^{-1}$."
Footnote 27. A table in the declaration presented the binding data for antibodies from every cell line. Values ranged from 13,867 to 125,204 cpm, and a substantial proportion of the antibodies showed binding greater than 50,000 cpm. In confirmation of Dr. Wand's statement, two antibodies with binding less than 25,000 cpm were found to have affinity constants greater than $10^{9}M^{-1}$.
Footnote 28. *See Rohm & Haas Co. v. Crystal Chem. Co* ., 722 F.2d 1556, 220 USPQ 98 (Fed. Cir. 1983).
Footnote 29. Even if we were to accept the PTO's 2.8% success rate, we would not be required to reach a conclusion of undue experimentation. Such a determination must be made in view of the circumstances of each case and cannot be made solely by reference to a particular numerical cutoff.
Footnote 30. *In re Strahilevitz* , 668 F.2d 1229, 1232, 212 USPQ 561, 563 (CCPA 1982).

Concurring/Dissenting Opinion Text

Concurrence/Dissent By:
Newman, J., concurring in part, dissenting in part.

A

I concur in the court's holding that additional samples of hybridoma cell lines that produce these high-affinity IgM monoclonal antibodies need not be deposited. This invention, as described by Wands, is not a selection of a few rare cells from many possible cells. To the contrary, Wands states that all monoclonally produced IgM antibodies to hepatitis B surface antigen have the desired high avidity and other favorable properties, and that all are readily preparable by now-standard techniques.
Wands states that his United States Patent No. 4,271,145 describes fully operable techniques, and is distinguished from his first four failed experiments that are referred to in the Rule 132 affidavit. Wands argues that these biotechnological mechanisms are relatively well understood and that the preparations can be routinely duplicated by those of skill in this art, as in *Hybritech, Inc. v. Monoclonal Antibodies, Inc* ., 802 F.2d 1367, 1380, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied* , 107 S.Ct. 1606 (1987). I agree that it is not necessary that there be a deposit of multiple exemplars of a cell system that is readily reproduced by known, specifically identified techniques.

B

I would affirm the board's holding that Wands has not complied with 35 U.S.C. §112, first paragraph, in that he has not provided data sufficient to support the breadth of his generic claims. Wands' claims on appeal include the following:
19. Monoclonal high affinity IgM antibodies immunoreactive with HBsAg determinants, wherein said antibodies are coupled to an insoluble solid phase, and wherein the binding affinity constant of said antibodies for said HBsAg determinants is at least $10^{9}M^{-1}$.
26. Monoclonal high affinity IgM antibodies immunoreactive with hepatitis B surface antigen.
Wands states that he obtained 143 "high binding monoclonal antibodies of the right specificity" in the successful fusions; although he does not state how they were determined to be high binding or of the right specificity, for Wands also states that only nine of these 143 were tested.
Of these nine, four (three from one fusion and one from another fusion) were found to have the claimed

high affinity and to be of the IgM isotype. Wands states that the other five were either of a different isotype or their affinities were not determined. (This latter statement also appears to contradict his statement that all 143 were "high binding".)
Wands argues that a "success rate of four out of nine", or 44.4%, is sufficient to support claims to the entire class. The Commissioner deems the success rate to be four out of 143, or 2.8%; to which Wands responds with statistical analysis as to how unlikely it is that Wands selected the only four out of 143 that worked. Wands did not, however, prove the right point. The question is whether Wands, by testing nine out of 143 (the Commissioner points out that the randomness of the sample was not established), and finding that four out of the nine had the desired properties, has provided sufficient experimental support for the breadth of the requested claims, in the context that "experi

ments in genetic engineering produce, at best, unpredictable results", quoting from *Ex parte Forman* , 230 USPQ 546, 547 (Bd.Pat.App. and Int. 1986).
The premise of the patent system is that an inventor, having taught the world something it didn't know, is encouraged to make the product available for public and commercial benefit, by governmental grant of the right to exclude others from practice of that which the inventor has disclosed. The boundary defining the excludable subject matter must be carefully set: it must protect the inventor, so that commercial development is encouraged; but the claims must be commensurate with the inventor's contribution. Thus the specification and claims must meet the requirements of 35 U.S.C. §112. *In re Fisher* , 427 F.2d 833, 839, 166 USPQ 18, 23-24 (CCPA 1970).
As the science of biotechnology matures the need for special accommodation, such as the deposit of cell lines or microorganisms, may diminish; but there remains the body of law and practice on the need for sufficient disclosure, including experimental data when appropriate, that reasonably support the scope of the requested claims. That law relates to the sufficiency of the description of the claimed invention, and if not satisfied by deposit, must independently meet the requirements of Section 112.
Wands is not claiming a particular, specified IgM antibody. He is claiming all such monoclonal antibodies in assay for hepatitis B surface antigen, based on his teaching that such antibodies have uniformly reproducible high avidity, free of the known disadvantages of IgM antibodies such as tendency to precipitate or aggregate. It is incumbent upon Wands to provide reasonable support for the proposed breadth of his claims. I agree with the Commissioner that four exemplars shown to have the desired properties, out of the 143, do not provide adequate support.
Wands argues that the law should not be "harsher" where routine experiments take a long time. However, what Wands is requesting is that the law be less harsh. As illustrated in extensive precedent on the question of how much experimentation is "undue", each case must be determined on its own facts. *See* , *e.g., W.L. Gore & Assocs., Inc. v. Garlock, Inc* ., 721 F.2d 1540, 1557, 220 USPQ 303, 316 (Fed. Cir. 1983), *cert. denied* , 469 U.S. 851 (1984); *In re Angstadt* , 537 F.2d 498, 504, 190 USPQ 214, 218 (CCPA 1976); *In re Cook* , 439 F.2d 730, 734-35, 169 USPQ 298, 302-03 (CCPA 1971).
The various criteria to be considered in determining whether undue experimentation is required are discussed in, for example, *Fields v. Conover* , 443 F.2d 1386, 170 USPQ 276 (CCPA 1971); *In re Rainer* , 347 F.2d 574, 146 USPQ 218 (CCPA 1965); *Ex parte Forman* , 230 USPQ at 547. Wands must provide sufficient data or authority to show that his results are reasonably predictable within the scope of the claimed generic invention, based on experiment and/or scientific theory. In my view he has not met this burden.

- End of Case -

ISSN 1526-8535

In re Marzocchi and Horton, 169 USPQ 367 (CCPA 1971)

In re Marzocchi and Horton

(CCPA)
169 USPQ 367

Decided Apr. 15, 1971
No. 8431
U.S. Court of Customs and Patent Appeals

Headnotes

PATENTS

1. Specification - Sufficiency of disclosure (§ 62.7)

Recitation of generic term "polyethyleneamine" must be taken as assertion by applicants that all of the "considerable number of compounds" which are included within generic term would, as a class, be operative to produce asserted enhancement of adhesion characteristics; Patent Office has no concern over breadth of term; its only relevant concern should be over truth of such assertion; first paragraph of 35 U.S.C. 112 requires nothing more than objective enablement; how such a teaching is set forth, either by use of illustrative examples or by broad terminology, is of no importance.

2. Pleading and practice in Patent Office - Rejections (§ 54.7)

Specification - Sufficiency of disclosure (§ 62.7)

Specification disclosure which contains teaching of manner and process of making and using the invention in terms corresponding in scope to those used in describing and defining subject matter sought to be patented must be taken as in compliance with enabling requirement of first paragraph of 35 U.S.C. 112 unless there is reason to doubt objective truth of statements contained therein which must be relied on for enabling support; assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that teaching contained in specification is truly enabling.

3. Pleading and practice in Patent Office - Rejections (§ 54.7)

Specification - Sufficiency of disclosure (§ 62.7)

In field of chemistry generally, there may be times when well-known unpredictability of chemical reactions will alone be enough to create reasonable doubt as to accuracy of broad statement put forward

as enabling support for claim; this will especially be the case where statement is, on its face, contrary to generally accepted scientific principles; most often, additional factors, such as teachings in pertinent references (not necessarily prior art), will be available to substantiate doubts that asserted scope of objective enablement is in fact commensurate with scope of protection sought and to support any demands based thereon for proof; it is incumbent upon Patent Office, whenever, a rejection on this basis is made, to explain why it doubts truth or accuracy of statement in supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with contested statement; otherwise, there would be no need for applicant to support his presumptively accurate disclosure.

Particular patents-Fiber Coatings

Marzocchi and Horton, Fiber Coatings - Nitrogen Compounds for Improving Adhesion of Vinyl Polymers to Glass, claims 6 and 12 of application allowed; claims 5 and 11 refused.

Case History and Disposition:

Page 367

Appeal from Board of Appeals of the Patent Office.

Application for patent of Alfred Marzocchi and Richard C. Horton, Serial No. 470,618, filed July 8, 1965; Patent Office Group 140. From decision rejecting claims 5, 6, 11, and 12, applicants appeal. Affirmed as to claims 5 and 11; reversed as to claims 6 and 12.

Attorneys:
Herman Hersh and McDougall, Hersh, Scott & Ladd, both of Chicago, Ill.

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(Staelin & Overman, Toledo, Ohio, and George A. Degnan, Washington, D. C., of counsel) for appellants.

S. Wm. Cochran (Fred W. Sherling of counsel) for Commissioner of Patents.

Judge:
Before Rich, Almond, Baldwin, and Lane, Associate Judges, and Durfee, Judge, United States Court of Claims, sitting by designation.

Opinion Text

Opinion By:
Baldwin, Judge.

This is an appeal from the decision of the Patent Office Board of Appeals which affirmed the final rejection of claims 5 and 11 of appellants' application under 35 U.S.C. 103 as unpatentable in view of

Werner [2] and of claims 6 and 12 under 35 U.S.C. 112 as being based on an inadequate disclosure. Claims 4 and 10 stand allowed.

The Invention

The subject matter of the claims on appeal involves a technique for improving the adhesion characteristics between glass fibers and vinyl polymer resins. Claim 5 is representative and reads as follows:

> 5. In the combination of glass fibers and a vinyl polymer resin composition present as a coating on the glass fiber surfaces, the improvement which comprises mixing the vinyl polymer resin, prior to coating of the glass fibers, with an amine compound in an amount corresponding to 2-10% by weight of the vinyl polymer resin, and in which the amine compound is monomeric vinyl pyrrolidone.

Claim 11 is drawn to the same concept as claim 5, but defines the invention as "a method of producing glass fibers coated with polyvinyl resin strongly bonded to the glass fiber surfaces." Claims 6 and 12 differ from claims 5 and 11 respectively solely in the recitation of "polyethyleneamine" as the critical "amine compound" additive.

The Section 103 Rejection

Claims 5 and 11 were rejected "as obvious in the sense of 35 U.S.C. 103 over Werner." Werner, the sole reference relied upon here, is addressed to the improvement in the bonding relationship between glass and polyvinyl halide resins. The pertinent disclosure is as follows [emphasis added]:

> I have found that polyvinyl halide resins may be successfully modified so as to obtain excellent glass adhesion by employing a mixture of a polyvinyl halide and a *polymer* of N-vinyl pyrrolidone. By employing a mixture containing from 80 to 97% of a polyvinyl halide and from 20 to 3% of a polymer of N-vinyl pyrrolidone, which term includes homopolymers of vinyl pyrrolidone and copolymers with other polymerizable monomers, a composition is obtained having extremely high adhesion to all glass surfaces.

On the basis of this teaching the examiner took the position, accepted by the board, that the claimed use of *monomeric* vinyl pyrrolidone rather than Werner's *polymeric* vinyl pyrrolidone would be obvious to one of ordinary skill in the art since Werner's teaching would indicate to "one skilled in the art * * * that it is the vinyl pyrrolidone moiety that is enhancing the adhesion." It was also suggested by the examiner that since the claims recite no temperature conditions for the coating operation and since monomers polymerize when heated, the claims could possibly cover circumstances wherein the monomer is polymerized during application. The board appears to have accepted this suggestion and to have extended it even further. It stated:

> All of Werner's examples specify heating at elevated temperatures (110°C.-130°C., 165°C., 325°F., 350°F.) with and without elevated pressures. Appellants' specification says nothing about retaining the vinyl pyrrolidone in monomeric form, much less anything about "maximizing adhesion" by preventing polymerization. Indeed, the very designation of the vinyl pyrrolidone as a "monomeric" material introduced into a polymer system for the purpose of altering the properties of such system implies subsequent polymerization of the monomer. Appellants' further argument that the monomer has entirely different capabilities and solubilities than the polymer is also unpersuasive.

Appellants' position on appeal in response to these assertions by the examiner and board is largely to

stress again the "marked difference between the properties and characteristics of a polymer as compared to a monomer," and to object to the "purely conjectural" assertion that the monomer polymerizes in the coating after it is applied. Additionally, appellants make the following contention:

> Even if it were assumed that appellants' monomeric vinyl pyrrolidone is polymerized when present in the polyvinyl chloride coating, there is no teaching or suggestion

> in Werner that the use of monomeric vinyl pyrrolidone has any efficacy whatsoever in compositions of the type disclosed and claimed. The basis suggested by the Patent Office for the rejection is tantamount to the allegation it would be "obvious to try" the monomer. This "test" of obviousness has been frequently repudiated by this court.

The sole issue is, of course, whether the Werner teaching does suggest to a person having ordinary skill in this art that the use of monomeric vinyl pyrrolidone would have the efficacy indicated in the appealed claims. We agree with appellants that whether the monomer polymerizes is irrelevant, at least in this regard. What is relevant, however, and here determinative, is the examiner's assertion that the Werner teaching would suggest that it is the vinyl pyrrolidone moiety alone and not some other characteristics peculiar to a polymer which is efficacious in producing the desired adhesion enhancement. [3] In the absence of anything to rebut this assertion, which is reasonable on its face, we are constrained to accept it as fact. The inferences which follow from such fact, i.e., that the monomer would possess this same characteristic and that one of ordinary skill would recognize such fact, are inescapable.

It is acknowledged that the above line of reasoning may be viewed as being tantamount to drawing the inference that, to one possessing the ordinary level of skill in this art, it would be "obvious to try" the monomer. Nevertheless, such an *inference of fact may,* at times, be enough to justify drawing the ultimate *conclusion of law* that the claimed subject matter as a whole would have been obvious under section 103. We are satisfied that the circumstances of this case justify an initial conclusion of obviousness. Since the record before us contains nothing to rebut that conclusion, the decision with regard to claims 5 and 11 must be affirmed.

The Section 112 Rejection

Claims 6 and 12, which recite the use of "polyethyleneamine" as the adhesion enhancer, were criticized by the examiner as being based on a disclosure which was not enabling under the first paragraph of 35 U.S.C. 112. The board affirmed his rejection of those claims with the following comment.

> The term is obviously generic to a considerable number of compounds varying in the number of ethylene groups, the number of amine groups and the relationship of the polyethylene groups to the amine groups, and accordingly does not provide a reasonable guide for those seeking to improve the adherence of vinyl resins to glass.

We will reverse the board's decision on this rejection since we are unable to find sufficient justification for the holding that appellants' disclosure is not enabling.

[1] Turning specifically to the objections noted by the board as indicated above, it appears that these comments indicate nothing more than a concern over the *breadth* of the disputed term. If we are correct, then the relevance of this concern escapes us. It has never been contended that appellants, when they included the disputed term in their specification, intended only to indicate a single compound. Accepting, therefore, that the term is a generic one, its recitation must be taken as an assertion by appellants that all of the "considerable number of compounds" which are included within the generic

term would, as a class, be operative to produce the asserted enhancement of adhesion characteristics. The only relevant concern of the Patent Office under these circumstances should be over the *truth* of any such assertion. The first paragraph of § 112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.

[2] As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejction can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling.

[3] In the field of chemistry generally, there may be times when the well-known

unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles. Most often, additional factors, such as the teachings in pertinent references, [4] will be available to substantiate any doubts that the asserted scope of objective enablement is in fact commensurate with the scope of protection sought and to support any demands based thereon for proof. In any event, it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure. Cf. In re Gazave, 54 CCPA 1524, 379 F.2d 973, 154 USPQ 92 (1967); In re Chilowsky, 43 CCPA 775, 229 F.2d 457, 108 USPQ 321 (1956).

In the present case, the circumstances we see do not support the reasonableness of any doubts which the Patent Office might have had concerning the adequacy of appellants' specification disclosure to support these claims. In fact, those circumstances tend to strengthen rather than weaken appellants' claim to the breadth of protection they seek. In the first place, it has not been asserted by the Patent Office that the chemical properties of known polyethyleneamines vary to such an extent that it would not be expected by one of ordinary skill in this art that any such compound would possess the necessary capability of enhancing adhesion. Additionally, we note that polyethyleneamine is listed in appellants' specification as being only one of a much larger class of amine compounds possessing this necessary characteristic. Finally, we recognize (as did the examiner) the generic nature of appellants' broader concept, i.e., that the desired property of adhesion enhancement stems largely from the amine moiety. It does appear that variation of certain of the secondary factors mentioned by the examiner, such as molecular weight or proportion of ethylene groups, might influence to some degree or even mask the essential "amine" property of the polyethylene amine or its obviously equally essential compatibility with vinyl polymers. However, we see no basis to conclude that the ready avoidance of this result would not be within the level of ordinary skill in this art. Compare In re Skrivan, 57 CCPA 1201, 427 F.2d 801, 166 USPQ 85 (1970).

Taking all these circumstances into consideration, we are constrained to conclude that the record before us contains insufficient grounds for questioning the accuracy of appellants' teaching that *any* polyethyleneamine (obviously excepting those whose essential "amine" characteristics and compatibility

with vinyl polymers would be masked by the secondary factors mentioned) will function to accomplish the asserted result. It follows that claims 6 and 12 must be held to be supported by a disclosure which is in compliance with the requirements of the first paragraph of 35 U.S.C. 112.

Summary

The decision of the board regarding claims 5 and 11 *affirmed;* that dealing with claims 6 and 12 is *reversed.*

Footnotes

Footnote 1. Serial No. 470,618, filed July 8, 1965, for "Fiber Coatings - Nitrogen Compounds for Improving Adhesion of Vinyl Polymers to Glass" as a continuation-in-part of Serial No. 96,106, filed March 16, 1961.

Footnote 2. U. S. Patent No. 2,853,465, issued September 23, 1958.

Footnote 3. Indeed, the reasonableness of such an assertion is confirmed by the very disclosure contained in appellants' application which indicates that efficacious adhesion enhancers are those "organic nitrogenous compounds which are characterized both by an organic constitution which is compatible with the vinyl polymers and by a polarity expressed in the nitrogen function." As also pointed out by appellants in their brief (about which more will be said later), the nature of the present invention resides in the use of *amine* compounds, broadly, as adhesion enhancers.

Footnote 4. Not necessarily *prior* art references, it should be noted, since the question would be regarding the *accuracy* of a statement in the specification, not whether that statement had been made before.

- End of Case -

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CMLS Cellular and Molecular Life Sciences

Review

Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology

G. M. Rossolini[a,*], S. Schippa[b], M. L. Riccio[a], F. Berlutti[b], L. E. Macaskie[c] and M. C. Thaller[d]

[a]Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Via Laterina 8, I-53100 Siena (Italy), Fax +39 577 263325, e-mail: Rossolini@unisi.it
[b]Istituto di Microbiologia, Università 'La Sapienza', Rome (Italy)
[c]School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT (UK)
[d]Dipartimento di Biologia, Cattedra di Microbiologia Applicata, Università di Roma 'Tor Vergata', Rome (Italy)

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Abstract. Bacterial nonspecific acid phosphohydrolases (NSAPs) are secreted enzymes, produced as soluble periplasmic proteins or as membrane-bound lipoproteins, that are usually able to dephosphorylate a broad array of structurally unrelated substrates and exhibit optimal catalytic activity at acidic to neutral pH values. Bacterial NSAPs are monomeric or oligomeric proteins containing polypeptide components with an M_r of 25–30 kDa. On the basis of amino acid sequence relatedness, three different molecular families of NSAPs can be distinguished, indicated as molecular class A, B and C, respectively. Members of each class share some common biophysical and functional features, but may also exhibit functional differences. NSAPs have been detected in several microbial taxa, and enzymes of different classes can be produced by the same bacterial species. Structural and phyletic relationships exist among the various bacterial NSAPs and some other bacterial and eucaryotic phosphohydrolases. Current knowledge on bacterial NSAPs is reviewed, together with analytical tools that may be useful for their characterization. An overview is also presented concerning the use of bacterial NSAPs in biotechnology.

Key words. Acid phosphohydrolases; bacteria; genetics; physiology; molecular evolution; microbial biotechnology.

Introduction

Bacteria have several enzymes able to dephosphorylate organic compounds, which play various essential or accessory roles in cell physiology. Most dephosphorylating reactions known to occur in the procaryotic cell involve the hydrolysis of phosphoester or phosphoanhydride bonds and are catalysed by a group of enzymes indicated overall as phosphohydrolases or phosphatases [1]. Some of these enzymes are secreted outside the plasma membrane, where they are either released in a soluble form or retained as membrane-bound proteins. These enzymes, which henceforth will be referred to as secreted phosphohydrolases, are believed to function essentially in scavenging organic phosphoesters (such as nucleotides, sugar phosphates, phytic acid etc.) that cannot cross the cytoplasmic membrane. Inorganic phosphate (Pi) and organic by-products are released,

* Corresponding author.

that can be transported across the membrane, thus providing the cell with essential nutrients [2–4]. Some secreted phosphohydrolases have evolved specialized functions relevant to microbial virulence (e.g. the respiratory burst-inhibiting acid phosphatases of *Legionella micdadei* [5] and *Francisella tularensis* [6], and the protein-tyrosine phosphatases of *Yersinia* spp. [7, 8] and *Salmonella enterica* ser. *typhimurium* [9]). Other phosphohydrolases are found in the cytosolic compartment, where they may be involved in dephosphorylating reactions occurring in signal transduction [10] as well as in several metabolic pathways.

The interest in bacterial phosphohydrolases is not only related to their multiple roles in the biology of the procaryotic cell and to their occasional involvement in microbial pathogenicity but also to the possibility of exploiting these enzymes as (i) investigative tools in enzymology (see, for instance, refs 11, 12) and in regulation of gene expression (see, for instance, refs 13, 14); (ii) paradigms for molecular evolution (see, for instance, refs 15, 16); (iii) markers for bacterial taxonomy and identification (see, for instance, refs 17–19); (iv) reporters in immunology and molecular biology (see, for instance, refs 20–21); (v) tools for bioremediation in environmental microbiology [22, 23].

Current knowledge on bacterial phosphohydrolases is far from being complete. Most of the available information is derived from studies performed in the *Escherichia coli* or *S. enterica* ser. *typhimurium* models of procaryotic cell, and even in these models, the exact number of phosphatase activities remains to be established and the roles of known enzymes are only partially understood [4]. Information on phosphohydrolases of other bacterial species is considerably more limited. Notions derived from comparative studies suggest that the phosphatase pattern can be variable even within closely related bacterial species [15, 24–27], so that results obtained studying one species may not necessarily be valid for others. The study of microbial phosphatase, therefore, remains an active investigational field, with relevance to various aspects of microbial physiology and biotechnology.

Classification of phosphatases, including the bacterial ones, was initially based on the biochemical and biophysical properties of the enzyme such as pH optimum (acid, neutral or alkaline), substrate profile (nonspecific vs. specific for certain substrates) and molecular size (high vs. low molecular weight). As molecular sequence data for different enzymes became available, it was recognized that, like other proteins, phosphatases could be grouped into different molecular families according to similarity at the level of primary structure. This structural criterion has led to the definition of various molecular families and superfamilies of phosphatases, and signature sequence patterns specific for each family have been identified [28] which are useful for a tentative identification of the function of newly discovered genes from large-scale sequencing projects.

The objective of this article is to review current information on bacterial nonspecific acid phosphohydrolases (NSAPs). This term refers to a group of secreted enzymes which are usually able to hydrolyse a broad array of structurally unrelated organic phosphoesters and exhibit optimal catalytic activity at acidic to neutral pH values. Some of these enzymes were purified and characterized several years ago [29–31], but only recently have further investigations provided additional insights concerning the structure, function and distribution of bacterial NSAPs [27, 32–42]. An overview is first presented, in which the research done on bacterial NSAPs is briefly outlined, and current knowledge on these enzymes is summarized. Analytical tools useful for studying bacterial NSAPs are then reviewed. A detailed description of known bacterial NSAPs follows, in which structural and functional features of the various enzymes, and their distribution, are reviewed and compared. The final section discusses the use of some bacterial NSAPs as tools for applications in biotechnology.

Bacterial NSAPs: an overview

The term 'NSAP' was originally adopted to indicate bacterial enzymes which, unlike alkaline phosphatase, show optimal catalytic activity at acidic to neutral pH values and, unlike specific phosphohydrolases (e.g. 3′-nucleotidases, 5′-nucleotidases, hexose-phosphatases and phytases), do not exhibit a marked substrate specificity, retaining activity towards several different and structurally unrelated phosphoesters.

The existence of NSAP activity in the *E. coli* periplasm was reported in the late sixties [43], but this enzyme was not purified to homogeneity and further characterized. The first bacterial NSAPs purified and characterized in detail were the periplasmic PhoN (or nonspecific acid phosphatase I) and AphA (or nonspecific acid phosphatase II) enzymes produced by *S. enterica* ser. *typhimurium* [29–32]. Both enzymes were made by polypeptides of relatively low molecular mass (around 25 kDa), but showed different biophysical and functional properties.

Subsequent studies, performed on different bacterial species, demonstrated that production of acid phosphohydrolases containing low molecular mass polypeptides (i.e. in the 25–30-kDa range) and showing properties similar to the *Salmonella* NSAPs was not restricted to members of the latter genus, being actually widespread among several different microbial taxa [27, 33, 36–40]. At the same time, cloning of some NSAP-encoding genes allowed identification, on the basis of amino acid

sequence relatedness, of the existence of two different molecular families of NSAPs that we proposed to designate as molecular class A and molecular class B bacterial NSAPs, respectively [37, 38]. According to this criterion, the *Salmonella* PhoN enzyme [34, 35] turned out to be a member of molecular class A, while the AphA enzyme belonged to molecular class B [37, 38].

Most recently, further investigation on NSAPs produced by nonenterobacterial species led to the discovery of a third molecular family of bacterial NSAP that we have proposed to designate as molecular class C [42]. Enzymes of this class appear to be distantly related to class B NSAPs from the structural and evolutionary standpoint, but unlike the latter, which are secreted across the cytoplasmic membrane, yielding soluble periplasmic proteins, they carry an amino-terminal signal sequence typical of bacterial lipoproteins and are found as membrane-bound lipoproteins.

Current knowledge on bacterial NSAPs can be summarized as follows: (i) bacterial NSAPs are widespread enzymes that can be found in several different microbial taxa; (ii) all the bacterial NSAPs thus far identified and characterized are secreted enzymes, of which some are produced as soluble periplasmic proteins, while others are membrane-bound lipoproteins; (iii) at least three different molecular families of bacterial NSAPs can be identified on the basis of relatedness at the sequence level, and members of these families are designated as molecular class A, B and C NSAPs, respectively; signature sequence motifs specific for each molecular class have been defined that can be useful for a tentative identification of new hypothetical proteins; in addition to sequence similarity, members of each molecular class share some common functional and biophysical features which can be exploited as phenotypic markers for presumptive classification of newly discovered enzymes; (iv) notwithstanding the existence of common features, members of each molecular class of NSAPs may exhibit functional differences suggesting that, within a molecular class, enzymes with different functions have evolved; in fact, although most NSAPs are active against a broad spectrum of substrates, some of them show a narrower substrate profile; (v) production of NSAPs of different molecular families can occur in the same bacterial species supporting the view that, at least in these cases, different physiological roles are played by enzymes of different classes; (vi) conserved structural motifs are shared among the various bacterial NSAPs and some other bacterial and eucaryotic phosphohydrolases, rendering the former enzymes interesting also for studies of molecular evolution and comparative enzymology.

Analytical tools for studying bacterial NSAPs

Enzyme assays with crude preparations

Screening for production of NSAP activity is complicated by the fact that the bacterial cell normally contains multiple phosphohydrolases with overlapping substrate profiles, whose production can be differentially regulated. Therefore, the simple measurement of phosphatase activity of whole cells or of crude cell extracts using chromogenic substrates is not expected to be highly informative. However, gross differences in the pattern of phosphatase production can also be detected using this simple approach [18, 26, 44], which is suitable for analysing a considerable number of strains. In fact, measurement of total phosphatase activity produced by different members of the family *Enterobacteriaceae* showed that *Providencia stuartii* and *Morganella morganii* are able to produce a high-level P_i-irrepressible acid phosphatase (HPAP) activity (indicated as HPAP phenotype), unlike most other enterobacterial species, which produce only low to moderate levels of acid phosphatase activity under similar conditions [18, 26]. This feature has also been exploited for rapid identification of the above species by means of suitable indicator media [18, 45].

Enzyme purification and characterization

The classical approach for characterization of individual bacterial phosphohydrolases is represented by enzyme purification followed by biophysical and biochemical characterization of the purified protein. This approach has been successfully pursued for the analysis of some NSAPs [29, 31] but can be rather complex and is not suitable for screening several strains. The purification procedure has to be adjusted for each new enzyme and may be complicated by the presence, in the starting material, of several enzymes active on the substrate used to monitor the purification steps.

Zymogram assays

A further alternative beyond enzymatic assays with crude preparations and enzyme purification is represented by the analysis of phosphatase activities using zymogram techniques. In this case crude extracts are first subjected to an electrophoretic separation, and phosphatase activities are subsequently detected in situ by means of chromogenic reactions. Such reactions are based either on substrates which yield coloured products upon dephosphorylation [27, 33, 36, 46] or on the detection of the released P_i by means of the acidified ammonium molybdate method, which yields a blue precipitate [37, 47].

Electrophoretic separation in zymograms is classically done under nondenaturing conditions, using either isoelectric focusing or gel electrophoresis. Although useful in separating the various activities, these procedures do not provide precise information on the molecular size of the enzyme. An interesting alternative is to conduct zymograms after sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [48] followed by a treatment which allows enzyme renaturation in the gel matrix (renaturing SDS-PAGE) [27, 36]. With this approach, proteins are separated on the basis of the size of their polypeptide component, and the migration distance at which the band of activity is detected depends on the molecular mass of the polypeptide component of the enzyme. Limitations of renaturing SDS-PAGE are represented by its intrinsic inability to detect heteropolymeric enzymes, and by the possibility that the boiling step in the presence of SDS may cause irreversible denaturation of the protein. According to our experience, NSAPs of all molecular classes (A, B and C) as well as other secreted bacterial phosphohydrolases, including alkaline phosphatases, 2′:3′-cyclic phosphodiesterases and acid-hexose phosphatases, can be zymographically detected after renaturing SDS-PAGE [27, 36–38, 41, 42, 49 and unpublished results].

Zymogram analysis is suitable in analysing several bacterial strains while providing significantly more information than simple enzymatic assays with crude preparations. Since different substrates can be used for the development of phosphatase activity, zymograms can be useful in determining the substrate profile of the various enzymes [27, 36, 49]. Moreover, enzyme inhibitors (e.g. EDTA, tartrate, fluoride ions, SDS etc.) can be added to the equilibration buffer at the desired concentration to assay their effect on the enzyme activity [27]. Densitometric analysis of the bands of activity can be employed for quantitative measurements that can be useful for comparing enzyme activity against different substrates or for studying regulation of production of the enzyme [36].

Expression-cloning of bacterial phosphatase-encoding genes

An alternative approach to studying bacterial phosphatases, which has proven to be invaluable for NSAPs, is that of expression-cloning of phosphatase-encoding genes followed by characterization of the cloned genes and their products [33, 37, 38, 42, 49, 50]. This approach is based on screening bacterial genomic libraries constructed in a multicopy plasmid vector for clones overproducing phosphatase activity detectable on plates of an indicator medium on which the host used for cloning has a phosphatase-negative phenotype. Of the various systems employed for expression-cloning of bacterial phosphatase-encoding genes, that based on the tryptose-phosphate phenolphtalein methyl green (TPMG) indicator medium has proven particularly useful, allowing the isolation of several different such genes [49] (table 1).

Class A bacterial acid phosphatases

Molecular class A acid phosphatases are a group of bacterial secreted phosphohydrolases which contain a polypeptide component with an M_r of 25 to 27 kDa and show conserved sequence motifs. Six different class A phosphatase-encoding genes have been cloned and sequenced (table 1, fig. 1), and their products have been characterized to various extents.

The *S. enterica* ser. *typhimurium* PhoN enzyme, also indicated as nonspecific acid phosphatase I, was the first class A enzyme purified and characterized in detail. PhoN-Se is a homodimeric protein containing two 27-kDa subunits. It is active against a very broad array of substrates including 3′- and 5′-nucleoside monophosphates, nucleoside diphosphates, nucleoside triphosphates, hexose and pentose phosphates, α- and β-glycerophosphate, *p*-nitrophenyl phosphate (*p*NPP), phenolphthalein diphosphate (PDP), α-naphthyl phosphate and pyrophosphate, but not diesters. Reaction velocities are similar overall for the various hydrolysable substrates. K_m values for the various substrates are in the 1–2 mM range. The pH optimum is around 5.5, using 5′-adenosine monophosphate (AMP) as substrate. PhoN-Se activity is inhibited by fluoride and mercuric ions, while being unaffected by EDTA and various other divalent cations including Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+} and Zn^{2+}. P_i partially inhibits enzyme activity at high concentrations (0.1 M), the inhibitory effect being more evident with 5′-nucleotides than with pNPP as substrates [29–31]. The *phoN-Se* gene was apparently acquired by *S. enterica* following a recent horizontal transfer of genetic material [35]. The gene is found in different *Salmonella* serovars, indicating that the transfer event occurred prior to diversification of the present-day salmonellae. In some strains the gene has been silenced by point mutations [35]. Expression of *phoN-Se* is under the control of the *phoP-phoQ* two-component regulatory system [51, 52], which promotes transcription of *phoN* and other PhoP-activated genes under low environmental Mg^{2+} concentrations [53]. Unlike other products of genes which are part of the *phoP-phoQ* regulon, PhoN-Se is not involved in *Salmonella* virulence [54].

The *Zymomonas mobilis* PhoC-Zm enzyme represents the major P_i-irrepressible acid phosphohydrolase produced by this species, and was the first sequenced class A enzyme [33]. The PhoC-Zm protein has not been purified and further characterized.

Table 1. NSAPs detected in bacteria.

Bacterial species (strain)[a]	NSAPs	Genes (EMBL accession #)[e]	References
Cedecea davisae (CIP 8034^T)	class A (subclass A1)[b]	-	27
Cedecea neteri (ATCC 33855^T)	class A (subclass A1)[b]	-	27
Chryseobacterium meningosepticum (CCUG 4310)	class C	*olpA* (Y12759)*	42
Citrobacter amalonaticus (ATCC 25405^T)	class B[b]	-	27
Citrobacter freundii (ATCC 8090^T)	class B[b]	-	27
Citrobacter koseri (CIP 7214)	class B[b]	-	27
Enterobacter aerogenes (CIP 6086^T)	class A (subclass A1)[b]	-	27
Enterobacter agglomerans (ATCC 29904)	-[c]		27
Enterobacter amnigenus (ATCC 33072^T)	-[c]		27
Enterobacter cloacae (CIP 6085^T)	-[c]		27
Enterobacter sakazaki (ATCC 29544^T)	-[c]		27
Enterobacter taylorae (ATCC 35317^T)	-[c]		27
Escherichia coli (MG1655)	class B	*aphA-Ec* (X86971)	27, 36, 41
Escherichia fergusonii (ATCC 35469^T)	class B[b]	-	27
Escherichia hermannii (ATCC 33650^T)	-[c]		27
Hafnia alvei (ATCC 29926)	class A (subclass A1)[b]	-	27
	class B[b]	-	27
Haemophilus influenzae (Rd)	class B	*napA-Hi* (Y07615)[f]	60
	class C	*hel* (M68502)	42, 62
Klebsiella oxytoca (CIP 666)	class A (subclass A1)[b]	-	27
Klebsiella planticola (CIP 8131)	class A (subclass A1)[b]	-	27
Klebsiella pneumoniae (CIP 52144)	class A (subclass A1)	*phoC-Kp**[g]	27
	class B[d]	*napA-Kp**[h]	27
Klebsiella terrigena (CIP 8007^T)	class A (subclass A1)[b]	-	27
Kluyvera ascorbata (ATCC 33434)	-[c]		27
Leclercia adecarboxylata (CIP100921)	-[c]		27
Leminorella grimontii (ATCC 33999^T)	-[c]		27
Moellerella wisconsensis (ATCC 35017^T)	-[c]		27
Morganella morganii (ATCC 25830^T)	class A (subclass A1)	*phoC-Mm* (X64444)*[i]	27, 37
	class B	*napA-Mm* (X78328)*[i]	27, 38
Proteus mirabilis (ATCC 29906^T)	class B[b]	-	27
Proteus penneri (ATCC 33519^T)	-[c]		27
Proteus vulgaris (ATCC 8427)	-[c]		27
Providencia alcalifaciens (CIP 5862)	class B[b]	-	27
Providencia rettgeri (ATCC 29944^T)	class B[b]	-	27
Providencia rustigianii (ATCC33673^T)	class B[b]	-	27
Providencia stuartii (ATCC 29914^T)	class A (subclass A1)	*phoN-Ps* (X64820)*[j]	27, 49
	class B[b]	-	27
Salmonella enterica ser. *typhi* (Ty2)	class A (subclass A2)[b]	-	27
	class B	*aphA-Se* (X96552)[k]	27
Salmonella enterica ser. *typhimurium* (LT2)	class A (subclass A2)	*phoN-Se* (X59036)	27, 29–31, 34, 35
	class B	-	31, 32
Serratia fonticola (CIP 7864^T)	-[c]		27
Serratia liquefaciens (CIP 674)	-[c]		27
Serratia marcescens (CIP 6755)	-[c]		27
Serratia odorifera (CIP 7901^T)	-[c]		27
Serratia plymutica (CIP 7712)	class A (subclass A2)[b]	-	27
Shigella flexneri (YSH 6000)	class A (subclass A1)	*phoN-Sf* (D82966)[l,m]	40
(clinical isolate, serotype 2a)	class A (subclass A3)	*apy – Sf* (U04539)[l,n]	39
(CIP 8248)	class B[b]	-	27
Yersinia enterocolitica (CIP 8027^T)	-[c]		27
Yersinia kristensenii (CIP 8030^T)	-[c]		27
Yersinia pseudotuberculosis (Yss133)	-[c]		27
Yokenella regensburgei (ATCC 35313)	class A (subclass A1)[b]	-	27
Zymomonas mobilis (CP4)	class A	*phoC-Zm* (M24141)	33

[a]CIP, Collection of the Institut Pasteur; ATCC, American Type Culture Collection; CCUG, Culture Collection of the University of Goteborg. [b]The enzyme has been detected in zymogram assays, and the class (and subclass) attribution was based on distinctive zymogram properties (see text for further details). The gene has not been cloned, nor has the protein been purified. [c]No NSAP activity was detected in zymogram assays performed as described in ref. 27. [d]The class B NSAP was not detectable in zymogram assays performed as described in ref. 27, either in this or in other *K. pneumoniae* strains including ATCC 13883^T. [e]Genes marked with an asterisk were isolated using the TPMG expression cloning procedure [49]. A minus sign indicates that the gene corresponding to the enzyme detected in zymograms or purified has not been cloned. [f]The accession number refers to the *napA-Hi* gene cloned and resequenced from strain CCUG 7317/A. [g]The gene was cloned from *K. pneumoniae* ATCC 13883^T (Passariello et al., unpublished results). [h]The gene was cloned from *K. pneumoniae* ATCC 13883^T. When expressed in *E. coli*, it yields a functional product (Passariello et al., unpublished results). [i]The gene was cloned from *M. morganii* RS12 [37]. [j]The gene was cloned from *P. stuartii* PV81 [49]. [k]The gene was cloned from *S. enterica* ser. *typhi* Sty4. [l]The gene is carried on the large virulence-associated plasmid. [m]Homologous plasmid-borne genes have also been detected in some clinical isolates of *Shigella* spp. and enteroinvasive *E. coli*. [n]An apyrase activity similar to that encoded by the *apy* gene has also been detected in other clinical isolates of *Shigella* spp. and enteroinvasive *E. coli*.

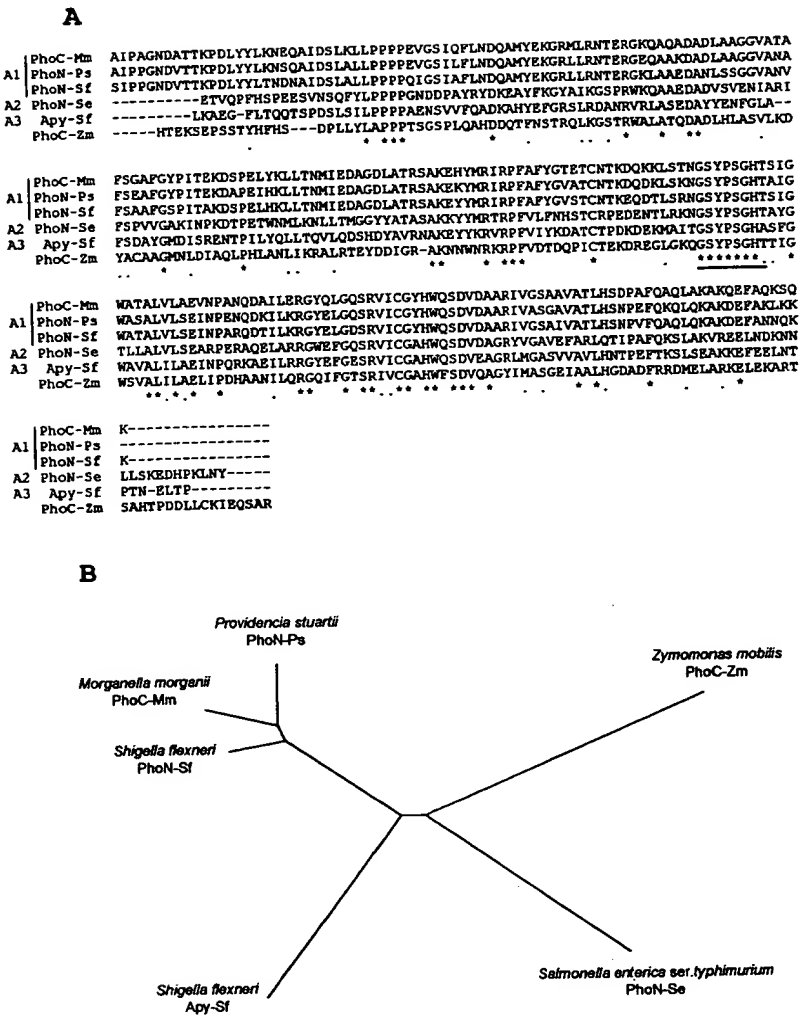

Figure 1. (*A*) Comparison of the amino acid sequences of the six known molecular class A NSAPs. The sequences of the mature proteins are reported. PhoC-Mm, PhoC protein of *M. morganii* [37]; PhoN-Ps, PhoN protein of *P. stuartii* [49, EMBL accession # X64820]; PhoN-Sf, PhoN protein of *S. flexneri* [40]; PhoN-Se, PhoN protein of *S. enterica* ser. *typhimurium* [35]; Apy-Sf, apyrase of *S. flexneri* [39]; PhoC-Zm, PhoC protein from *Z. mobilis* [33]. The proposed distinction in subclasses A1, A2 and A3 is also indicated. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The region corresponding to the signature sequence motif for this family of enzymes is indicated by a horizontal bar. (*B*) Unrooted tree showing phylogenetic relationships among the various class A proteins.

The two class A genes of *M. morganii* (*phoC-Mm*) and *P. stuartii* (*phoN-Ps*) were shotgun-cloned via the TPMG expression-cloning procedure while studying the molecular bases of the HPAP phenotype exhibited by isolates of these species [37, 49]. In *Morganella* isolates the class A enzyme represents the major P_i-irrepressible acid phosphatase activity, and the HPAP phenotype is associated with PhoC-Mm production. In this species, the presence of PhoC is apparently able to prevent induction of alkaline phosphatase when PhoC-hydrolysable organic phosphosters represent the sole phosphate source in the medium, probably as a consequence of PhoC-mediated P_i release from the substrate [37]. Although not specifically investigated, a similar scenario is likely to occur also in *P. stuartii*. The *Morganella* PhoC-Mm enzyme has been purified and characterized. PhoC-Mm is a homotetrameric protein containing four 25-kDa subunits. It exhibits a broad substrate specificity including 5'- and 3'-nucleoside monophosphates, glucose 6-phosphate, β-glycerophos-

phate and aryl-phosphates (*p*NPP and PDP), but not diesters. The highest reaction velocities are observed with 5'-nucleotides, glucose 6-phosphate and aryl-phosphates. The pH optimum is around 6, using *p*NPP as substrate. PhoC-Mm activity is not inhibited by EDTA, tartrate or fluoride, and is only slightly inhibited by high (0.1 M) P_i concentrations [37]. The *P. stuartii* class A enzyme has not been purified and characterized in detail. However, being very similar to that of *M. morganii* at the sequence level (84% of identical amino acid residues, fig. 1) it is expected to retain similar properties; partial characterization of this enzyme by means of zymograms yielded results consistent with this hypothesis [27, 49]. The occurrence of these highly homologous class A genes in more than one member of the enterocluster 3 lineage [55], along with their values of G + C contents which are consistent with those of the respective species, suggest that these genes are vertically derived from a common ancestor present in the corresponding lineage before divergence of the above species. Analysis of sequence data also suggests that the *S. typhimurium phoN-Se* gene was not acquired from any the above species. In fact, only *P. stuartii*, which has a low G + C content both at the genomic level (41%) [56] and in its own class A gene (43%), could have been a suitable donor candidate, given the low (43%) G + C content of the *Salmonella phoN* gene [35]. In this case, however, a significantly higher degree of similarity with the *Salmonella* gene would have been expected for the *P. stuartii* than for the *M. morganii* allele, and an overall higher degree of similarity would also have been expected between the *Salmonella* gene and those carried by the two members of enterocluster 3.

Considering that the degree of sequence divergence between the class A enzyme of *Salmonella* and those of *M. morganii* and *P. stuartii* is substantially higher than that between the two latter proteins (fig. 1), and that the *Salmonella* enzyme also differs from them as far as quaternary structure (homodimeric vs. homotetrameric) and susceptibility to fluoride (susceptible vs. resistant) are concerned, we have proposed to further distinguish class A enzymes into at least two subclasses indicated as A1 (prototype enzyme: PhoC-Mm) and A2 (prototype enzyme: PhoN-Se) [27].

The two class A enzymes found in *S. flexneri*, PhoN-Sf and Apy-Sf, are both encoded by genes carried on the large virulence-associated plasmid harboured by clinical isolates of this species [39, 40].

The *Shigella* PhoN-Sf protein is an NSAP which exhibits a broad substrate profile including nucleotides (like the *Morganella* class A enzyme, PhoN-Sf appears to be more active on 5'-nucleotides than on 3'-nucleotides), *p*NPP, glucose 6-phosphate and *β*-glycerophosphate. The pH optimum is at 6.6. The enzyme activity is not inhibited by chelators of divalent ions (EDTA, *o*-phenanthroline), fluoride, tartrate, cysteine, L-phenylalanine, L-tryptophan, benzamidine and soybean trypsin inhibitor, while being inhibited by *N*-bromosuccinimide, dithiothreitol and diisopropylfluorophosphate, suggesting that serine and tryptophan residues, as well as disulphide bonds, are relevant to PhoN-Sf activity [40]. At the sequence level, it exhibits a higher degree of similarity to the *M. morganii* and *P. stuartii* class A enzymes than to the other members of this class (fig. 1). This enzyme, therefore, can be classified as a subclass A1 NSAP. The PhoN-Sf protein is produced by only some *Shigella* and enteroinvasive *E. coli* (EIEC) strains, and is apparently not involved in the virulence phenotype of these bacteria [40].

The *Shigella* Apy-Sf protein shows some distinctive features as compared with the other class A enzymes. The native Apy-Sf enzyme is a 25-kDa monomer. It exhibits a marked preferential activity on nucleoside triphosphates (NTPs), which are hydrolysed sequentially to the corresponding diphosphates and monophosphates through release of P_i. It is also active on pyrophosphate and, although to a lower extent, on *p*NPP, but not on AMP. This enzyme can therefore be considered essentially as an ATP diphosphohydrolase or apyrase (EC 3.6.1.5). The optimal pH value for activity is between 7 and 7.5 [39]. Similarly to the other class A enzymes, the Apy-Sf activity is not inhibited by EDTA, while it is inhibited by fluoride (like enzymes of subclass A2), *o*-vanadate, sodium azide and various divalent cations including Ba^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} [39]. Considering the peculiar functional and structural features of the Apy protein, along with the degree of divergence observed at the sequence level with the other class A enzymes (fig. 1), we propose to distinguish a further molecular subclass for class A enzymes, subclass A3 (prototype enzyme: Apy-Sf). The *apy-Sf* gene or closely related alleles are carried by virulent *Shigella* spp. and enteroinvasive *E. coli* strains and are expressed in a thermoregulated manner [39], like many other virulence-associated genes of *Shigella* [57]. This observation, together with the localization of the enzyme in the periplasmic space, the specific activity of the enzyme on NTPs and the dramatic decrease of the NTPs pool in eucaryotic cells invaded by *Shigella* [58], suggests that Apy-Sf could be involved in the virulence phenotype of these pathogens [39, 58].

Comparison of the amino acid sequences of the six known class A enzymes shows the existence of various conserved domains (fig. 1), and a signature sequence motif for this family of enzymes has been defined as G-S-Y-P-S-G-H-T (PROSITE PDOC00891; [28]). This motif was defined before the sequence of the Apy-Sf enzyme was available. Considering also the latter enzyme, the class A acid phosphatase signature motif

could be modified as G-S-Y-P-S-G-H-[TA]. The existence of a conserved sequence motif, K-X(6)-R-P-X(12,54)-P-S-G-H-X(31,54)-S-R-X(5)-H-X(2)-D, among class A enzymes, a neutral phosphatase of *Treponema denticola*, some lipid phosphatases (including bacterial phosphatidylglycerol phosphate phosphatases and mammalian phosphatidic acid phosphatases), mammalian glucose-6-phosphatases and a yeast diacylglycerol pyrophosphatase, has recently been identified, suggesting that all these enzymes could be mechanistically related and that the conserved residues are likely essential for enzyme function and possibly part of the catalytic site [59].

Class A acid phosphatases belonging to subclasses A1 and A2 can be zymographically detected by renaturing SDS-PAGE using various substrates, including the chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), or PDP in combination with methyl-green [27]. When renaturing SDS-PAGE is used for zymogram detection of these enzymes, BCIP (which

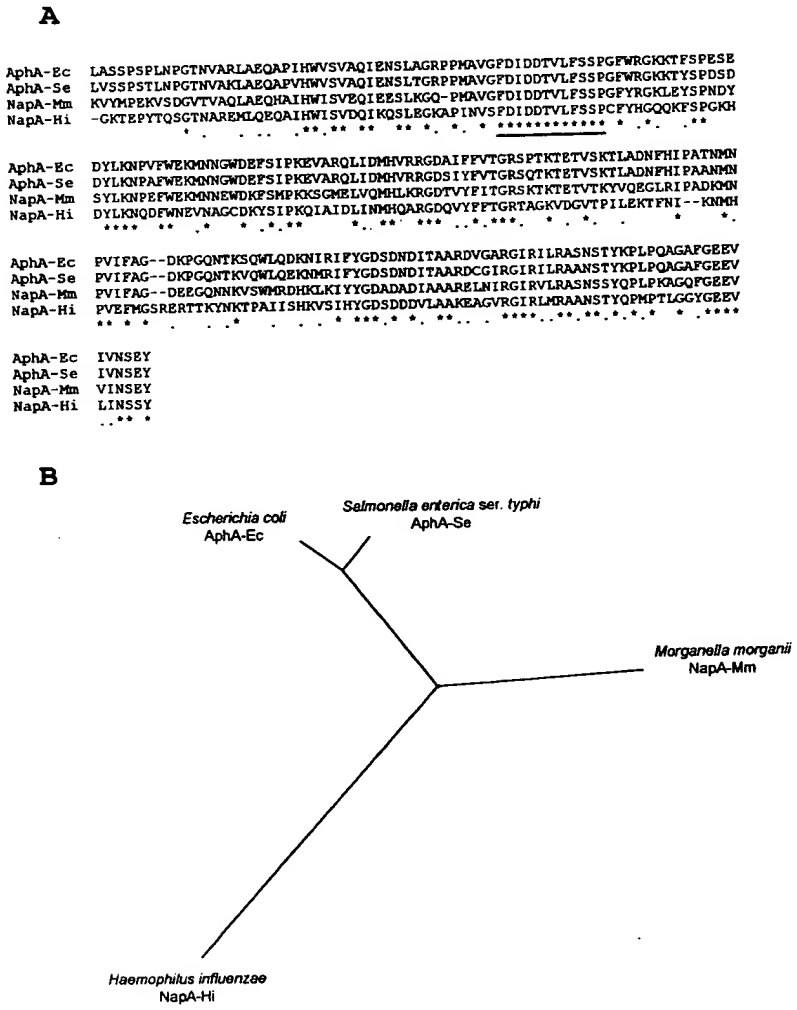

Figure 2. (*A*) Comparison of the amino acid sequences of the four known molecular class B NSAPs. The sequences of the mature proteins are reported. AphA-Ec, AphA protein of *E. coli* [41]; AphA-Se, AphA protein of *S. enterica* ser. *typhi* [EMBL accession # X96552]; NapA-Mm, NapA protein of *M. morganii* [38]; NapA-Hi, NapA protein of *H. influenzae* (EMBL accession # Y07615). Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The region corresponding to the proposed signature sequence motif for this family of enzymes is indicated by a horizontal bar. (*B*) Unrooted tree showing phylogenetic relationships among the various class B proteins.

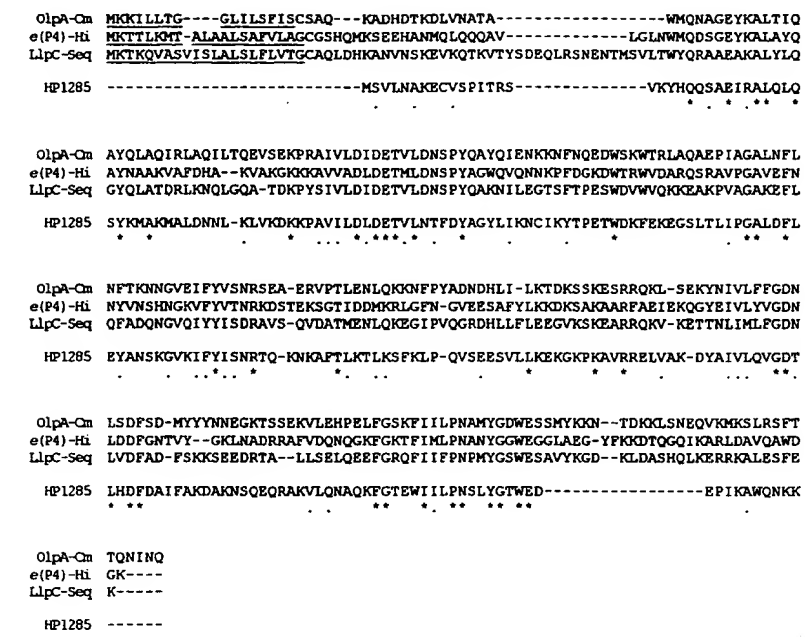

Figure 3. Comparison of the amino acid sequences of known or putative molecular class C NSAPs. The sequences of the native proteins are reported. OlpA-Cm, OlpA protein of *C. meningosepticum* [42; EMBL accession # Y12759]; *e*(P4)-Hi, *e*(P4) outer membrane lipoprotein of *H. influenzae* [62]; LlpC-Seq, LlpC membrane lipoprotein of *S. equisimilis* [63]. The sequence of the hypothetical protein of *H. pylori* (HP1285) [64] showing significant similarity with class C NSAPs is also shown. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. The lipoproteins signal peptides are underscored.

apparently is not hydrolysed by molecular class B NSAPs, see below) is useful for their differentiation from class B enzymes, which contain a polypeptide component of similar size. Additional features useful in differentiating class A from class B activities in zymograms are represented by resistance to inhibition by EDTA, and inactivation by low SDS concentrations. Resistance to inhibition by fluoride can be tested to differentiate enzymes of subclass A1 from those of subclass A2 [27].

A screening of representative strains of various *Enterobacteriaceae* for the presence of subclass A1 and A2 NSAPs, conducted using zymograms after renaturing SDS-PAGE, showed that production of similar enzymes is not restricted to *S. enterica* ser. *typhimurium*, *M. morganii*, *P. stuartii* or *Shigella* strains carrying the virulence plasmid. In fact, production of a putative subclass A1 NSAP was also detected in representative strains of *Cedecea* spp., *Enterobacter aerogenes*, *Hafnia alvei*, *Klebsiella* spp. and *Yokenella regensburgei*, while production of a putative subclass A2 NSAP was also detected in a *Serratia plymuthica* strain (table 1). In the same study no such enzymes were detected in representative strains of *Citrobacter* spp., *Enterobacter* spp. other than *E. aerogenes*, *Escherichia* spp., *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Leminorella grimontii*, *Moellerella wisconsensis*, *Proteus* spp., *Providencia* spp. other than *P. stuartii*, *Serratia* spp. other than *S. plymuthica* and *Yersinia* spp. (table 1). However, since it cannot be excluded that the growth conditions might have been nonpermissive for enzyme production in some species, a confirmation for the above class A-negative patterns should be sought, both under different growth conditions, and at the genetic level.

According to current knowledge on class A NSAP distribution in enteric bacteria, enzymes of subclass A1 appear to be scattered among various enterobacterial lineages, while the occurrence of enzymes of subclass A2 seems to be rather exceptional. This scenario suggests that subclass A1 genes have circulated among members of some lineages during enterobacterial evolution. The finding of a subclass A1 gene (*phoN-Sf*) on the large plasmid carried by some *Shigella* and EIEC strains [40] actually raises the possibility that other subclass A1 genes could be also located on extrachromosomal elements, and that similar alleles could have been exchanged among the ancestors of some enterobacterial species via plasmid-mediated transfer. On the

other hand, the rare subclass A2 genes found in enteric bacteria would have been acquired by recent horizontal transfer from nonenterobacterial members, their phylogeny being distinct from that of subclass A1 alleles. The more specialized *apy-Sf* gene (proposed as a member of a new subclass) carried by the large virulence-associated plasmid of *Shigella* and EIEC strains [39] may have been acquired during plasmid evolution or evolved as a virulence-associated gene from a plasmid-borne subclass A1 ancestor following a duplication event. The contemporary presence of the *phoN-Sf* gene on the same plasmid could represent the trace of a similar event.

Apart from the *S. flexneri* apyrase, which has evolved a rather specific substrate profile and for which a role in microbial pathogenicity has been hypothesized, the physiological roles of the other class A NSAPs remain to be established.

Class B bacterial acid phosphatases

Molecular class B acid phosphatases include a group of secreted bacterial phosphohydrolases which contain a polypeptide component with an M_r of approximately 25 kDa and share conserved sequence motifs. Although the polypeptide size is similar to that of class A NSAPs, class B enzymes are completely unrelated to the former at the sequence level.

In their native form, class B NSAPs are 100-kDa homotetrameric proteins comprising four polypeptide subunits. Unlike polymeric class A NSAPs, class B enzymes tend to be quite resistant to depolymerization by SDS and, in SDS-PAGE, migrate at least in part as 100-kDa bands if the sample, prepared in Laemmli's buffer [48], is not subjected to the boiling treatment [27, 32, 38, 41]. Unlike class A NSAPs, class B enzymes are apparently unable to dephosphorylate the chromogenic substrate BCIP, retain their activity in the presence of low SDS concentrations and are inhibited by EDTA [27, 31, 38, 41]. These features can be exploited for putative identification of class B NSAPs in zymograms following renaturing SDS-PAGE [27].

Four different class B phosphatase-encoding genes have been cloned and sequenced (table 1, fig. 2), and their products have been characterized to various extents.

The *S. enterica* AphA-Se enzyme was the first class B NSAP purified and characterized in detail [31, 32]. It was originally purified from *S. enterica* ser. *typhimurium* LT2 and was also named nonspecific acid phosphatase II [31] to distinguish it from the class A NSAP (PhoN-Se, also named nonspecific acid phosphatase I) which had already been identified in this strain [29, 30]. The AphA-Se enzyme is active on various organic phosphomonoesters, including 5'- and 3'-uridine monophosphate (UMP), *p*NPP and α-naphthyl phosphate, but not on diesters. The highest-reaction velocities are observed with 3'-nucleotides and *p*NPP. The pH optimum for the phosphatase activity is in the acidic range and appears to be substrate-dependent, being lower (5 to 5.5) with 3'-UMP or *p*NPP and higher (around 6.5) with 5'-UMP. The K_m value of the enzyme for 5'-UMP was calculated to be 0.3 mM. The phosphatase activity of the AphA-Se enzyme is inhibited by EDTA, by high P_i concentrations (50% of activity reduction in the presence of 0.1 M of P_i), and by nucleosides. The inhibitory effect of nucleosides is higher with 2'-deoxyribonucleosides than with the corresponding ribonucleosides, is evident even at low concentrations (28–79% of activity reduction, depending on the nucleoside type, in the presence of a 0.1 mM concentration), and appears to be concentration-dependent [31, 32]. The AphA-Se enzyme is also able to function as a phosphotransferase if suitable organic compounds carrying a free hydroxyl group are present as phosphate acceptors together with a hydrolysable phosphoester which can function as a phosphate donor. This low-energy phosphotransferase activity was demonstrated using *p*NPP as a phosphate donor and either alkylalcohols (methanol, ethanol, ethylene glycol or glycerol) at high concentrations (0.2 to 2 M) or nucleosides at low concentrations (0.1 mM) as phosphate acceptors. With alcohols, the transphosphorylation rate increases by increasing the acceptor concentration, and the transphosphorylation activity is associated with an increase of the *p*NPP-splitting activity, without affecting the rate of release of P_i. With nucleosides as acceptors, the transphosphorylation reaction is seen at thousandfold lower concentrations and is not associated with an increase but actually a decrease of the *p*NPP-splitting activity (due to the inhibitory effect of nucleosides on the enzyme activity), so that nucleosides appear to be more effective than alcohols as phosphate acceptors. The products of transphosphorylation of nucleosides are mostly represented by 3'-nucleotides, with only minor amounts of 5'- and 2'-nucleotides [32]. The AphA-Se enzyme tends to adhere to plastic and glass surfaces, but the immobilized protein is inactive. This phenomenon accounts for the apparently low stability of the enzyme in diluted solutions, and can be prevented by the presence of nonionic detergents (such as Triton X-100 or *n*-octyl glucoside) or of polyethylene glycol 6000 over a broad concentration range. Nonionic detergents are also able to redissolve and reactivate the immobilized enzyme when present at concentrations near or above their critical micelle value [32]. Crystals of the purified AphA-Se enzyme have also been obtained [32], but the three-dimensional structure of the protein has not been solved. The gene encoding the AphA-Se enzyme has recently been cloned from a *S. enterica* ser. *typhi* strain (table 1). Polymerase chain reaction (PCR) amplification of the corresponding genetic locus from *S. enterica* ser. *typhimurium* LT2 and

restriction analysis of the amplimer demonstrated that the *aphA* genes carried by the two serovars are highly conserved at the sequence level (M. C. Thaller et al., unpublished results).

The *M. morganii* NapA-Mm enzyme was the first class B NSAP to be cloned and sequenced [38]. NapA-Mm was initially identified as a minor P_i-irrepressible NSAP produced by this species, in addition to the major P_i-irrepressible PhoC-Mm enzyme [37, 38]. Characterization of the NapA-Mm protein purified from an *E. coli* strain carrying the cloned *napA-Mm* gene showed that its biophysical and functional properties were similar to those of the *S. enterica* AphA-Se enzyme, and consequently the definition of molecular class B NSAPs was proposed, with the NapA-Mm sequence being the prototypic one [38]. The *Morganella* NapA-Mm enzyme is active on various organic phosphomonoesters, including 5'- and 3'-nucleoside monophosphates, aryl-phosphates (*p*NPP and PDP), β-glycerophosphate and sugar phosphates (glucose 6-phosphate and ribose 5-phosphate), but not on diesters. The highest reaction velocities are observed with purine nucleotides, *p*NPP and PDP. With *p*NPP as substrate, the pH optimum of the phosphatase activity is about 6 [38]. Substrate-dependency of the pH optimum has not been investigated with this enzyme. The phosphatase activity of the NapA-Mm enzyme is inhibited by EDTA, by high P_i concentrations (P_i does not decrease enzyme activity up to a 20 mM concentration, while a partial inhibitory effect becomes apparent at higher P_i concentrations), by Ca^{2+} and by nucleosides and 2'-deoxynucleosides (31–63% of activity reduction, depending on the nucleoside type, in the presence of a 0.1 mM concentration). The phosphatase activity of the NapA-Mm enzyme is unaffected by tartrate and fluoride and stimulated by low concentrations (1 mM) of Mg^{2+}, Co^{2+} and Zn^{2+}. Similarly to the *Salmonella* AphA-Se enzyme, NapA-Mm is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols or nucleosides as phosphate acceptors. Although not studied in comparable detail, transphosphorylation properties of the NapA-Mm enzyme were similar, overall, to those of the *Salmonella* class B enzyme [38].

In *E. coli*, the presence of a periplasmic acid phosphatase with features typical of a class B NSAP was initially detected by zymogram assays [27, 36]. As soon as the sequence of the *Morganella* class B enzyme became available, an unknown open reading frame, located in the *tyrB-uvrA* intergenic region (at approximately 92 min of the genetic map) of the *E. coli* chromosome was putatively identified as the gene encoding this enzyme on the basis of the sequence similarity of its product with the *Morganella* NapA-Mm protein [37]. The identity of this gene, named *aphA-Ec*, was subsequently confirmed by cloning and expression experiments [41]. The *E. coli* class B NSAP was purified from an *E. coli* strain engineered for overexpression of the *aphA-Ec* gene. Its biophysical and functional properties are similar, overall, to those of the *S. enterica* AphA-Se enzyme and of the *M. morganii* NapA-Mm enzyme. The *E. coli* AphA-Ec enzyme is active against a broad array of organic phosphomonoesters, including 5'- and 3'-nucleoside monophosphates, aryl-phosphates (*p*NPP, PDP, phenyl phosphate and *O*-phospho-L-tyrosine), nonaromatic phospho-amino acids (*O*-phospho-L-serine and *O*-phospho-L-threonine), β-glycerophosphate, ribose 5-phosphate and phytic acid, showing the highest reaction velocities with aryl-phosphates and nucleotides. No activity was detectable against adenosine triphosphate (ATP), glucose 1-phosphate, glucose 6-phosphate or diesters. Similarly to the *Salmonella* AphA-Se enzyme, the pH optimum for the phosphatase activity of the *E. coli* class B NSAP is around 6–6.5 for 5'-nucleoside monophosphates and lower (5.5–6) for *p*NPP. The phosphatase activity of the NapA-Mm enzyme is inhibited by EDTA, by P_i (in this case a slight decrease of the enzyme activity is evident at 5 mM P_i concentration and increases progressively with the P_i concentration), by Ca^{2+} and by nucleosides (67–80% of reduction of activity, depending on the nucleoside type, in the presence of a 0.1 mM concentration). The phosphatase activity of the AphA-Ec enzyme is unaffected by fluoride and stimulated by low concentrations of Mg^{2+}. Similarly to the other class B enzymes, AphA-Ec is also able to function as a phosphotransferase using *p*NPP as a phosphate donor and either alkylalcohols at high concentrations or nucleosides at low concentrations as phosphate acceptors [41]. In *E. coli*, production of the AphA-Ec enzyme is detectable when cells are grown on carbon sources other than glucose, being undetectable when glucose is available as a carbon source [36].

In *H. influenzae*, the presence of a chromosomal gene encoding a hypothetical protein similar to other class B NSAPs was identified at complement of nucleotides 511018–510313 [60]. In the original sequence data this open reading frame was interrupted by frameshifts, which were solved after cloning and resequencing of the corresponding region (fig. 2). The *H. influenzae* class B gene was named *napA-Hi*. When subcloned into an *E. coli* expression vector, the *napA-Hi* gene was actually able to direct production of a recombinant protein endowed with acid phosphatase activity and showing zymogram properties typical of class B NSAPs (G. M. Rossolini et al., unpublished results).

Molecular class B NSAPs appear to be quite conserved at the sequence level, the percent of identical amino acid residues ranging from 91%, when comparing the *E. coli* and *Salmonella* proteins, to 46% when comparing the enzymes from *Enterobacteriaceae* to that of *Haemo-*

philus (fig. 2). Comparison of amino acid sequences shows the existence of various highly conserved domains. The sequence motif F-D-I-D-D-T-V-L-F-S-S-P could be proposed as a signature sequence pattern for bacterial class B NSAPs (fig. 2). At the sequence level, class B NSAPs also appear to be distantly related to molecular class C bacterial NSAPs and to some plant acid phosphatases (see below).

Class B acid phosphatases can be zymographically detected after renaturing SDS-PAGE using various substrates, including the chromogenic substrate PDP in combination with methyl green [27, 36, 38, 41]. Distinctive features useful in identifying class B enzymes in zymograms performed following renaturing SDS-PAGE have been previously described (see above).

A screening of representative strains of various *Enterobacteriaceae* for the presence of class B acid phosphatases, performed by renaturing SDS-PAGE, showed that production of similar enzymes is not restricted to *S. enterica*, *M. morganii* or *E. coli*. In fact, production of a putative class B NSAP was also detected in representative strains of *Citrobacter* spp., *Escherichia fergusonii*, *Hafnia alvei*, *Proteus mirabilis*, *Providencia* spp. and *Shigella* spp. (table 1). In the same study, no class B enzymes were detected in representative strains of *Cedecea* spp., *Enterobacter* spp., *Escherichia hermanii*, *Klebsiella* spp., *Kluyvera ascorbata*, *Leclercia adecarboxylata*, *Leminorella grimontii*, *Moellerella wisconsensis*, *Proteus* spp. other than *P. mirabilis*, *Serratia* spp., *Yersinia* spp. and *Yokenella regensburgei* (table 1). However, since production of class B NSAPs can be regulated [36], the class B-negative zymogram pattern observed in some species could also have resulted from growth conditions nonpermissive for enzyme production at levels detectable by the zymogram assay. This point is being currently investigated by searching the genomic DNAs of strains that showed a class B-negative zymogram pattern for the presence of class B genes by means of polymerase chain reaction (PCR) amplification using degenerate primers for two highly conserved regions of known class B NSAPs. Preliminary results suggest that class B alleles are also carried by at least some of the species showing a class B-negative zymogram pattern (M. C. Thaller et al., unpublished results). Moreover, a class B NSAP-encoding gene has been cloned from *K. pneumoniae* via the TPMG expression-cloning procedure (table 1), indicating that, in this species, a class B gene is actually present and can also be functional.

The widespread distribution of class B alleles among enteric bacteria suggests that a class B gene was likely present in the enterobacterial ancestor or was acquired early in the lineage. During subsequent evolution of *Enterobacteriaceae*, this gene may have undergone mutations or rearrangements accounting for the present-day distribution and expression pattern. The phylogeny of class B NSAPs in enteric bacteria, therefore, appears to be substantially different from that of class A NSAPs.

Concerning the physiological role of class B NSAPs, it was initially proposed that in *S. enterica* ser. *typhimurium* the AphA-Se enzyme could represent the major periplasmic 5'-nucleotide-splitting enzyme, and possibly also substitute for alkaline phosphatase which is lacking in this species. This proposal was based both on the results of physiological studies performed with some mutants (altough not genetically characterized) and on kinetic data (a relatively low K_m value for 5'-UMP), and was also supported by the knowledge that, as compared to *E. coli*, *S. enterica* ser. *typhimurium* is lacking a counterpart for both the UshA periplasmic 5'-nucleotidase and the PhoA alkaline phosphatase [31]. However, it was recently shown that most *S. enterica* serovars other than *typhimurium* do produce a functional UshA homologue [24], and that production of a class B acid phosphatase is not restricted to *Salmonella* but also occurs in *E. coli* and in several other enterobacterial species [27], including those able to produce 5'-nucleotidase and alkaline phosphatase activities [15, 25]. This updated knowledge on enterobacterial periplasmic phosphatases, therefore, would suggest reconsideration of the above hypothesis, leaving the physiological function of class B enzymes an open issue.

Class C acid phosphatases and the superfamily of DDDD phosphohydrolases

Molecular class C acid phosphatases have recently been identified as a group of secreted bacterial lipoproteins endowed with NSAP activity that contain a polypeptide component with an M_r of approximately 30 kDa and share conserved sequence motifs. At the sequence level class C enzymes appear to be related, although distantly, to class B NSAPs and also to some plant acid phosphohydrolases.

The first identified class C NSAP was the OlpA enzyme of *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* which, among the ex-flavobacterial species, is the most relevant from the clinical standpoint [61]. This enzyme was discovered as a zymographically detectable NSAP activity containing an approximately 30-kDa polypeptide, while screening nonenterobacterial species for the presence of NSAPs [42]. The gene encoding OlpA was isolated from a genomic library of *C. meningosepticum* CCUG 4310 via the TPMG expression-cloning procedure, and sequence analysis yielded a protein whose primary structure did not resemble either class A or class B NSAPs, and contained a signal peptide typical of bacterial lipo-

proteins (fig. 3). Consequently, the existence of a new molecular class of bacterial NSAPs (molecular class C) was proposed, with OlpA-Cm being the prototype enzyme of this class [42].

OlpA-Cm was found to share significant sequence similarity with two other bacterial lipoproteins for which a phosphatase activity had not been previously demonstrated: the *e*(P4) outer membrane lipoprotein of *H. influenzae* [62], and a cytoplasmic membrane lipoprotein of *Streptococcus equisimilis* [63] (fig. 3). Cloning and expression of the *H. influenzae* gene (*hel*) encoding the *e*(P4) lipoprotein in *E. coli* has recently confirmed that this lipoprotein also exhibits acid phosphatase activity against various phosphomonoesters [42]; hence *e*(P4) can be classified also as a member of class C NSAPs. Inclusion of the *S. equisimilis* LlpC membrane lipoprotein into the family of molecular class C NSAPs is awaiting the demonstration of a NSAP activity of the above protein.

Comparison of the amino acid sequences of known or putative class C NSAPs allowed identifying various conserved domains (fig. 3). An overall sequence similarity was also observed between these proteins and a hypothetical secreted protein encoded by an open reading frame (HP1285) located at complement of nucleotides 1362349–1361660 of the *Helicobacter pylori* chromosome [64], which could represent another member of this molecular family (fig. 3).

The recent discovery of class C NSAPs has not allowed enough time for a detailed analysis of their enzymatic properties. Concerning the physiological role, the *e*(P4) lipoprotein of *H. influenzae* was recently demonstrated to be essential for haemin uptake by this species [65]. The relationship between this function, which is carried out by a domain located near the amino-terminus of the protein that contains sequences putatively involved in haemin binding and/or transport [65], and the NSAP activity of the protein remains to be clarified.

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AphA-Ec     65-VGFDIDDTVLFSSPGFWRGKKTFSPESEDYLKNPVFWEKMNNGWDEFSIPKEVARQLIDMHV
AphA-Se     64-VGFDIDDTVLFSSPGFWRGKKTYSPDSDDYLKNPAFWEKMNNGWDEFSIPKEVARQLIDMHV
NapA-Mm     64-VGFDIDDTVLFSSPGFYRGKLEYSPNDYSYLKNPEFWEKMNNEWDKFSMPKKSGMELVQMHL
NapA-Hi     63-VSFDIDDTVLFSSPCFYHGQQKFSPGKHDYLKNQDFWNEVNAGCDKYSIPKQIAIDLINMHQ

OlpA-Cm     71-IVLDIDETVLDNSP--YQAYQIENKKNF----NQEDWSKWTRLAQAEPIA--GALNFLNFTK
e(p4)-Hi    80-IVADLDETMLDNSP--YAGWQVQNNKPF----DGKDWTRWVDARQSRAVP--GAVEFNNYVN
LlpC-Seq    95-IVLDIDETVLDNSP--YQAKNILEGTSF----TPESWDVWVQKKEAKPVA--GAKEFLQFAD
  HP1285    53-VILDLDETVLNTFD--YAGYLIKNCIKY----TPETWDKFEKEGSLTLIP--GALDFLEYAN

APS1-Lyce  105-WIFDVDETLLSNLP--YYSDHRYGLEVF----DDVEFDKWVENGTAPALG--SSLKLYQEVL
AP-Glymax  100-WVFDIDETTLSNLP--YYADHGFGVELY----NETSFNKWVDLGEAPALP--ESLKLYKKLL
                  *.*.* *                                          .

AphA-Ec        RRGDAIFFVTGRSPTKTETVSKTLADNFHIPATNMNPVIFA------G--DKPGQNTKSQWL
AphA-Se        RRGDSIYFVTGRSQTKTETVSKTLADNFHIPAANMNPVIFA------G--DKPGQNTKVQWL
NapA-Mm        KRGDTVYFITGRSKTKTETVTKYVQEGLRIPADKMNPVIFA------G--DEEGQNNKVSWM
NapA-Hi        ARGDQVYFFTGRTAGKVDGVTPILEKTFNI--KNMHPVEFM------GSRERTTKYNKTPAI

OlpA-Cm        NNGVEIFYVSNRSEA-ERVPTLENLQKKNFPYADNDHLI-L-------KTDKSSKESRRQKL
e(P4)-Hi       SHNGKVFYVTNRKDSTEKSGTIDDMKRLGFN-GVEESAFYL-------KKDKSAKAARFAEI
LlpC-Seq       QNGVQIYYISDRAVS-QVDATMENLQKEGIPVQGRDHLLFL-------EEGVKSKEARRQKV
  HP1285       SKGVKIFYISNRTQK-NKAFTLKTLKSFKLP-QVSEESVLL-------KEKGKPKAVRRELV

APS1-Lyce      KLGFKVFLLTGRSER-HRSVTVE--NLMNAGFHDWHKLILR-GSDDHGKTATTYKSERRNAM
AP-Glymax      SLGIKIVFITGRPLD-QKAVTATNLNLKLAGYHTWEKLITKNTSEYHGKTAVTYKSTERKKL
                  . . . * .                                          . .

AphA-Ec        QDKNIRI--FYGDSDNDI-40
AphA-Se        QEKNMRI--FYGDSDNDI-40
NapA-Mm        RDHKLKI--YYGDADADI-40
NapA-Hi        ISHKVSI--HYGDSDDDV-40

OlpA-Cm        -SEKYNIVLFFGDNLSDF-72
e(P4)-Hi       EKQGYEIVLYVGDNLDDF-68
LlpC-Seq       -KETTNLIMLFGDNLVDF-65
  HP1285       -AKDYAIVLQVGDTLHDF-52

APS1-Lyce      VEEGFRIVGNSGDQWSDL-20
AP-Glymax      EEKGYKIIGNIGDQWSDL-20
                     .   **   *
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Figure 4. Comparison of the amino acid sequences of known or putative molecular class C NSAPs with those of class B NSAPs and of two plant acid phosphatases. APS1-Lyce, tomato acid phosphatase [Swiss-Prot accession # P27061]; AP-Glymax, soybean acid phosphatase [EMBL accession # AJ223074]; for the names of other sequences see legends to figs 2 and 3. Identical residues are indicated by an asterisk; conservative amino acid substitutions are indicated by a dot. Only the relevant protein domains are shown in this alignment; numbers at the beginning of each sequence indicate the number of residues from the N-terminus of the native protein; numbers at the end of each sequence indicate the number of residues from the C-terminus of the protein.

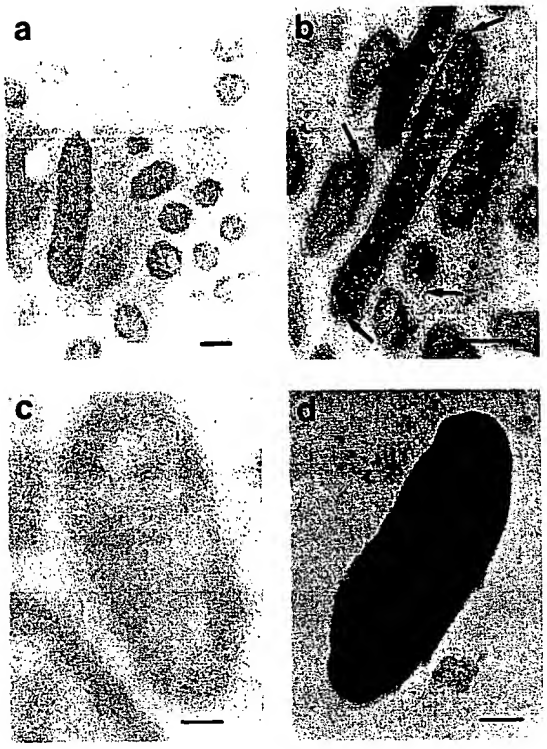

Figure 5. Phosphatase production and uranyl phosphate accumulation by the N14 strain. (*a*, b) Immunogold labelling to show phosphatase production and localization [86]. (a) Cells of a phosphatase deficient mutant show no immunogold label. (b) The parent strain shows surface and periplasmically localized enzyme (arrowed). The use of cell sections shows negligible intracellular enzyme. Bars are 500 nm. (*c*, d) Uranium accumulation by whole cells in the presence of UO_2^{2+} and the phosphatase substrate glycerol 2-phosphate. (*c*) Uranium uptake by the phosphatase deficient mutant or by the parent strain in the absence of UO_2^{2+} (control). (*d*) Uranium uptake by the parent strain following exposure to UO_2^{2+} for several hours. The accumulated precipitate was identified as HUO_2PO_4 using energy dispersive X-ray analysis, proton induced X-ray emission analysis, infrared spectroscopy, solid-state magic angle spinning ^{31}P NMR and X-ray powder diffraction analysis [23, 77, 82].

Comparison of class C enzymes with other sequenced proteins allowed identifying conserved sequence motifs between the former enzymes and other bacterial or eucaryotic proteins, including class B bacterial NSAPs and some plant acid phosphatases (fig. 4). These findings suggest that class B and class C bacterial NSAPs, together with their plant homologues, are members of the same superfamily of phosphohydrolases that we propose to indicate as "DDDD" after the presence of four invariant aspartate residues within the most conserved domains (fig. 4). All these enzymes could be mechanistically and phylogenetically related, and the highly conserved residues are likely essential for enzyme function and could be part of the catalytic site.

Bacterial NSAPs as tools in biotechnology

Similarly to alkaline phosphatase, which has been successfully used as a reporter in enzyme immunoassays [20] or as a probe for protein topology [21], bacterial NSAPs could also be exploitable for biotechnological applications. To date, this objective has been pursued with some class A NSAPs that have been successfully used for biotechnological applications as outlined below.

Use of NSAPs as tools for environmental bioremediation

In 1982 an environmental Gram-negative, rod-shaped, oxidase-negative, fermenting strain (N14), identified as *Citrobacter* sp., which was able to tolerate cadmium and accumulate it when grown in the presence of Cd, was isolated from metal-polluted soil [66]. Such a potential was present in cells pregrown in the absence of the metal, and was retained by cells subsequently exposed to metals, either in a resting or immobilized state [67, 68]. The requirement for the presence of suitable organic phosphoesters for metal accumulation to occur, along with the time-dependent metal accumulation, and the consistency of metal uptake with high-level production of a periplasmic acid phosphatase activity by the N14 strain, suggested an enzyme-mediated metal-uptake mechanism possibly involving the cleavage of the phosphoester bond to yield inorganic phosphate which precipitates stoichiometrically with available heavy metal cations, so that the metal phosphates are tightly bound as $MHPO_4$ (M = metal) at the cell surface [67, 69, 70]. This hypothesis was verified by X-ray microanalysis and magic angle spinning ^{31}P nuclear magnetic resonance (NMR) analysis: the heavy metal precipitation occurs initially at discrete loci at the cell surface of resting cells, being followed by a heavy cellular deposition of the same material [71]. Moreover, a phosphatase-deficient mutant of the N14 strain (lp4a) was ineffective at accumulation of heavy metals [72, 73].

Further studies demonstrated an effective phosphatase-mediated accumulation, by the N14 strain, of various heavy metals and actinides, including Am, Pu and U, in the form of their insoluble hydrogen phosphates or phosphates (fig. 5) [22, 72, 74–77]. Removal of Th(IV) is poor per se but can be facilitated if La(III) is also incorporated into the solution, and the same method is effective in enhancing removal of Pu(IV) [78]. The product of uranyl biocrystallization, cell-bound hydrogen uranyl phosphate (HUP) [23, 77], is a polycrystalline, lamellar material with intercalative

cation-exchange ability (i.e. cations of other metals could displace protons from within the interlamellar space) [79–82]. In contrast to other heavy metals, Ni is not removed by the metal phosphate deposition reaction, but the intercalative ion exchange property of HUP could be successfully exploited in the removal of Ni from dilute aqueous solutions, since Ni^{2+} ions are reversibly incorporated into cell-bound HUP to form nickel uranyl phosphate $[Ni(UO_2PO_4)_2]$. This was designated as microbially enhanced chemisorption of heavy metals [80–82].

Removal of heavy metals from aqueous wastes via microbially generated precipitant ligands ('biomineralization'), whose formation is dependent on the production of a secreted acid phosphatase, has therefore become a valuable alternative to classical and biosorptive methods of waste water treatment [72, 74, 83–85].

Purification of the acid phosphatase produced by strain N14 and determination of the amino-terminal sequence and of some internal amino acid sequences showed significant similarities with known class A NSAPs [86], suggesting that similar enzymes could be involved in the biomineralization process. In fact, an *E. coli* strain engineered to overproduce the *Salmonella* class A NSAP demonstrated a high efficiency for accumulating uranyl ions and acting as a bioorganic ion exchanger via the accumulation of HUP and subsequent removal of Ni (G. Baskanova et al., unpublished). This finding represents the first example of an acid phosphatase-mediated metal biomineralization process by a microorganism other than N14, and opens the possibility of future engineering of improved strains for specific industrial applications.

Use of NSAP-encoding genes as insertional inactivation targets in cloning vectors

Class A NSAP-encoding genes have been exploited as targets for insertional inactivation in cloning vectors that allow direct identification of recombinants. Using these vectors, recombinants are easily identified on the basis of their phosphatase-negative phenotype, while clones containing an empty vector exhibit a phosphatase-positive phenotype [87, 88].

The major advantages of similar vectors, as compared to the most popular *lacZ* α-complementation-based cloning vehicles (i.e. the pUC series and derivatives [89, 90]), are represented by the possibility of using them in any *E. coli* host, independently on its *lac* genotype, and by the significantly lower cost of the indicator medium as compared with that used for the β-galactosidase plate assay.

The problem of engineering a versatile multiple cloning site (MCS) into the phosphatase gene, without disturbing the activity of its product, has been solved by replacing the region encoding the phosphatase signal peptide with a modified amino-terminal moiety of the *E. coli lacZ* gene, derived from a *lacZ* α-complementation-based cloning vector [88]. With this approach, cloning vectors have been constructed that allow identification of recombinants based on phosphatase inactivation while retaining all the MCS facilities that made so popular the *lacZ* α-complementation-based vectors [88].

Concluding remarks and future work

Although the existence of bacterial NSAPs has been known for a relatively long time, only recently has knowledge on these enzymes undergone a considerable advancement concerning their primary structure, genetics, enzymology, distribution and phylogeny. Notwithstanding this advanced knowledge, the role of NSAPs in microbial physiology remains an open issue, at least for most of them. Indeed, the widespread distribution and high-level conservation of some of these enzymes (e.g. the molecular class B NSAPs in enteric bacteria) suggest an involvement in functions relevant to procaryotic cell physiology, but these functions have yet to be identified with certainty. On the other hand, contemporaneous production of NSAPs of different molecular classes by some bacteria (e.g. production of both a class A and a class B enzyme by *M. morganii*, *H. alvei*, *P. stuartii* and *S. enterica*, or production of both a class B and a class C enzyme in *H. influenzae*) suggests that, at least in such instances, NSAPs of different classes play different roles. To understand this essential point, additional investigations will be required, concerning various fundamental aspects. An evaluation of the kinetic parameters of these enzymes could be useful to determine their catalytic efficiencies toward different hydrolysable substrates. In fact, kinetic parameters have not been investigated for most NSAPs, while the approximate functional data available for several enzymes are often not fully comparable due to differences in the experimental conditions adopted. A comprehensive comparative analysis of the functional properties of NSAPs of each molecular class would also be interesting for understanding the molecular evolution and structure-function relationships of these enzymes. Regulation of the various NSAP-encoding genes, and the effects of gene inactivation or overexpression on cell physiology, represent additional aspects that would deserve investigation to understand the physiological role of these enzymes.

Another interesting topic for additional investigation is represented by production of NSAPs throughout the microbial kingdom. In fact, all NSAPs thus far de-

scribed are from representative strains of enteric bacteria or of a few other Gram-negative species (with the exception of the hypothetical class C enzyme of *S. equisimilis*). Identification and characterization of NSAPs from other bacterial taxa would help at understanding their role, while providing valuable information in the field of bacterial and molecular evolution. Concerning the potential utility of bacterial NSAPs in the sector of applied microbiology and biotechnology, the successful exploitation of some class A NSAPs for similar purposes should encourage further investigation in this field, also with enzymes of other molecular families.

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Purification and Characterization of Two Phytases from *Escherichia coli*

R. Greiner, U. Konietzny, and Kl.-D. Jany[1]
Institute of Nutritional Physiology, Federal Research Centre for Nutrition, Karlsruhe, Federal Republic of Germany

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Two periplasmatic phytases, called P1 and P2, were purified about 16,500-fold to an apparent homogeneity with a recovery of 7 and 18%, respectively. The enzymes behave as monomeric proteins with molecular masses of about 42 kDa. Because of the limited amounts recovered, the amino terminal sequence of only one of the phytases was determined. Both enzymes are very specific for phytate and have little or no activity on other phosphate esters tested. The kinetic parameters for the hydrolysis of Na-phytate and *p*-nitrophenyl phosphate are k_{cat}/K_M 478 $\times 10^5\ s^{-1}\ M^{-1}$ and $0.6 \cdot 10^5\ s^{-1}\ M^{-1}$ at pH 4.5. The hydrolysis pathway for phytate was elucidated for P_2; consequently, this enzyme is a 6-phytase. The chemical and kinetic properties of the purified phytase P2 points to an identity with an enzyme described by Dassa *et al.* (1982, *J. Biol. Chem.* 257, 6669–6676) as a pH 2.5 acid phosphatase. Because of the kinetic parameters it would be better to denote this enzyme as a phytase. © 1993 Academic Press, Inc.

Phytic acid [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate][2] is the major storage form of phosphorus in seeds and pollen (2). Because of its strong chelating properties, phytic acid is regarded as an antinutritive factor. Phytic acid forms insoluble complexes with nutritionally important metals such as calcium, zinc, magnesium, and iron, thereby decreasing their bioavailability (3, 4). Phytase (*myo*-inositol hexaphosphate phosphohydrolase) hydrolyzes phytic acid to *myo*-inositol and phosphoric acid. Two types of phytases are known: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26), indicating the predominant attack of the susceptible phosphoester bond. Phytases are present in plants, certain animal tissues, and microorganisms. They have been studied most intensively in seeds of plants such as wheat, barley, bean, corn, soybean, rice, and cotton (5, 6). Phytase activity in microorganisms has been found most frequently in fungi, in particular Aspergilli (7, 8). It occurs also in bacteria (9), yeasts (10), and rumen microorganisms (11). There is only one report on the occurrence of phytase activity in *Escherichia coli*, but without any characterization of the enzyme(s) (12). Four acid phosphatases have been identified in the periplasmatic space of *E. coli*. These enzymes can be distinguished from each other by their pH optima and their substrate specificities (1, 13, 14).

Phytases are of interest for biotechnological applications, especially for the reduction of phytate in food and feedstuff. Supplementation of animal feedstuff with phytases will increase the bioavailability of phosphate, thus decreasing phosphorus pollution in areas of intensive animal agriculture. The addition of phytases will diminish antinutritional effects of food having a high content of phytate. We have purified and characterized two phytases from *E. coli* cells grown in the late stationary phase.

MATERIALS AND METHODS

Chemicals. *E. coli* K12 (ATCC 33965) and most of the enzyme substrates were purchased from E. Merck (Darmstadt, Germany). Phytic acid dodecasodium salt was from Aldrich (Steinheim, Germany). All other chemicals were products of Fluka, Boehringer-Mannheim, Serva, and Sigma. CM-Sepharose CL 6B, DEAE-Sepharose CL 6B, Phenyl-Sepharose CL 4B, high-load 16/60 Sephacryl S-200 HR, and Mono S HR 5/5 were obtained from Pharmacia (Freiburg, Germany). Ultrasep ES 100 RP18 was purchased from Bischoff (Leonberg, Germany). All reagents were of analytical grade.

Growth of the bacteria. Bacteria were grown aerobically in 2-liter flasks containing 500 ml LB-medium, pH 7.5, at 37°C. Cell growth was followed at 578 nm. Anaerobiosis was obtained by layering paraffin oil at the surface of the growth medium and stopping agitation.

Protein estimation. Total protein concentration was determined by Coomassie blue G-250 dye-binding using bovine serum albumin as a standard (15).

[1] To whom correspondence should be addressed at Federal Research Centre for Nutrition, Engesserstrasse 20, D-7500 Karlsruhe 1, Germany. Fax: 0049 (0) 7247/22820.

[2] Abbreviations used: PNPP, *p*-nitrophenyl phosphate; PMSF, phenylmethylsulfonyl-flouride; EDC, N-(dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; IP_x, *myo*-inositol phosphate derivative; TBAH, tetrabutylammonium hydroxide; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Assay of phytase and acid phosphatase. Phytase and acid phosphatase measurements were carried out at 35°C. The enzymatic reactions were started by the addition of 10 µl enzyme to the assay mixtures.

The incubation mixture for phytase determination consisted of 350 µl 0.1 M sodium acetate, pH 4.5, containing 500 nmol sodium phytate. After an incubation time of 30 min, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (16). One and one-half milliliters of a freshly prepared solution of acetone: 5N H_2SO_4:10 mM ammonium molybdate (2:1:1, v/v) and 100 µl 1.0 M citric acid were added to the assay mixture. Any cloudiness was removed by centrifugation prior to the measurement of the absorbance at 355 nm. In order to calculate the enzyme activity a calibration curve was performed over the range of 5 to 600 nmol inorganic phosphate. The activity (U) was expressed as 1 µmol phosphate liberated per minute.

Acid phosphatase was determined in 200 µl 0.25 M glycine–HCl, pH 2.5, or 0.1 M sodium acetate, pH 4.5, containing 25 µmol PNPP. After 15 min the reaction was stopped with 1.0 ml 1 N NaOH. The activity of the acid phosphatase was determined by measuring the absorbance of the formed *p*-nitrophenolate at 405 nm. One unit of enzyme was defined as the amount of acid phosphatase releasing 1 µmol *p*-nitrophenolate per minute. The enzymatic hydrolysis of other phosphorylated compounds was tested as described under phytase activity, but sodium phytate was substituted by 1 µmol of the phosphorylated compounds.

Effect of cations and potential inhibitors on enzyme activity. To investigate the effect of cations and potential inhibitors on enzyme activity, these substances were preincubated with the phytase 15 min at 37°C before performing the standard phytase assay. The reaction with 0.1 mM EDC was performed in 12.5 mM $Na_4P_2O_7$ pH 5.0, for 30 min at RT.

Identification of the hydrolysis products. Enzyme and Na-phytate were incubated as described for the activity determination but with the phosphate assay omitted. From the incubation mixture samples (50 µl) were removed periodically and the reaction was stopped by heat treatment (90°C, 5 min). Twenty microliters of the samples was chromatographed on Ultrasep ES 100 RP18 (2 × 250 mm). The column was run with 0.4 ml/min of an eluant consisting of formic acid:methanol:water: TBAH (44:56:5:1.5, v/v), pH 4.25, essentially as described by Sandberg *et al.* (17). Analysis of the stereoisomers was performed with the same samples according to the method of Mayr (18).

Purification of the phytases. All operations were carried out at 0 to 4°C, unless otherwise stated. Bacteria were suspended in 20 mM sodium acetate, pH 4.5, and sonicated for 3 × 60 s. The cell debris was removed by centrifugation at 12,000*g* for 30 min and the clear supernatant was used for the ammonium sulfate precipitation at 25–80% saturation. The protein material obtained was dialyzed against 20 mM sodium acetate, pH 4.5. Any precipitate formed was removed by centrifugation at 10,000*g* for 30 min.

CM-Sepharose CL 6B chromatography. The dialyzed ammonium sulfate fraction was loaded onto a CM-Sepharose CL 6B column (3.5 × 20 cm) equilibrated with 20 mM sodium acetate, pH 4.5. The column was washed with 300 ml of the same buffer and then the proteins bound were eluted with a linear gradient from 0 to 1.0 M NaCl (2000 ml) in 20 mM sodium acetate, pH 4.5. The fractions containing phytase activity were pooled and dialyzed against 20 mM Tris–HCl, pH 7.8.

DEAE-Sepharose CL 6B chromatography. The dialyzed active pool was loaded onto a DEAE-Sepharose CL 6B column (4 × 15 cm) equilibrated with 20 mM Tris–HCl, pH 7.8. After eluting the unbound inactive protein from the column with equilibration buffer, a linear gradient of 0 to 0.5 M NaCl (1000 ml) in 20 mM Tris–HCl, pH 7.8, was applied. The fractions containing phytase activity were pooled and dialyzed against 0.1 M sodium acetate, pH 5.0.

Phenyl-Sepharose CL 4B chromatography. The pooled fraction from the previous step was saturated to a final concentration of 1.0 M ammonium sulfate and applied on a phenyl-Sepharose CL 4B column (3 × 30 cm) equilibrated with 0.1 M sodium acetate, pH 5.0, containing 1.0 M ammonium sulfate. The column was washed with equilibration buffer and the proteins were then eluted with a linear gradient from 1.0 to 0 M ammonium sulfate (2000 ml) in 0.1 sodium acetate, pH 5.0. Two peaks with phytase activity were separated (Fig. 2A). The second one was taken for further purification. It was dialyzed against 20 mM sodium acetate, pH 4.5. All columns were run at a flow rate of 50 ml/h, and 10-ml fractions were collected.

Mono S HR 5/5 chromatography. The dialyzed pool of the phenyl-Sepharose column was applied onto a Mono S HR 5/5 column equilibrated with 20 mM sodium acetate, pH 4.5. First the column was washed with the same buffer for 30 min and then with 0 to 0.5 M NaCl in 20 mM sodium acetate, pH 4.5, for 90 min. The collecting of fractions was carried out manually corresponding to the protein profile at 280 nm.

Gel electrophoresis. Native gel electrophoresis was carried out with 7.5% gels at pH 8.3 (19, 20) and with 5% gels at pH 4.8 (21). Enzymatic staining of the protein was performed with 1-naphthylphosphate coupled with Fast Blue B in 0.1 M sodium acetate, pH 4.5, in the dark (22). SDS-electrophoresis using 10% gels was performed according to Laemmli (19). Gels were stained with Coomassie brilliant blue R-250 or by silver staining (23).

Gel-filtration. To assess the molecular mass of the native phytases the purified proteins were gel-filtered on 16/60 Sephacryl S-200 HR equilibrated with 50 mM Tris-acetate, pH 6.2, 0.2 M NaCl. The column was calibrated with glucose-6-phosphate dehydrogenase, M_r 120,000; creatine kinase, M_r 81,000; bovine serum albumin, M_r 68,000; β-lactoglobulin, M_r 40,000; and myoglobin, M_r 17,000.

Amino acid analysis. Amino acid analysis was performed on a Biotronik LC 6000 analyzer. The protein samples were hydrolyzed in 6 N HCl in sealed and evacuated tubes at 108°C for 24 and 96 h. Sequence determination was carried out with an Applied Biosystem 470 A/120A protein sequencer using the standard program according to the manufacturer.

Comparison with the pH 2.5 acid phosphatase. The determination of the properties for the comparison of the phytase P2 with the pH 2.5 acid phosphatase was carried out as described by Dassa *et al.* (1).

RESULTS

Localization and Production of the Phytases

No phytase activity was detectable in the culture media of growing *E. coli* cells; consequently, there was no question of an extracellular phytase. By the osmotic shock procedure, 90% of the phytase activity and an 80-fold higher specific activity was found in comparison to the sonicated extract. Therefore the *E. coli* phytases must be regarded as periplasmatic enzymes.

In normal growing cells only a very low phytase activity was detectable. However, the activity increased markedly when the cells ran into the stationary phase. A further increase of the activity was obtained after a shift from aerobic to anaerobic conditions. The maximum specific activity of the phytases was obtained 4 h after the beginning of its accumulation (Fig. 1). Therefore, cells grown under anaerobic conditions and harvested in the late stationary phase were used as enzyme source.

Purification of the Phytase

A summary of the purification scheme is given in Table I. The phytase activity was eluted as a single sharp activity peak from each soft-gel ion-exchange column after application of the gradient. No splitting of the phytase activity was obtained but most of the inactive material was removed by this procedure.

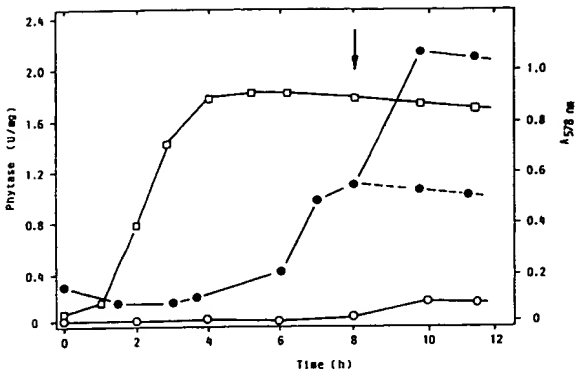


FIG. 1. Phytase and acid phosphatase activity during the growth of *E. coli* in LB-media at 37°C. (● – ●) phytase under aerobic conditions, (● -- ●) phytase under anaerobic conditions, (○) acid phosphatase, (□) cell density at 578 nm. The arrow indicates the change from aerobic to anaerobic conditions.

During the hydrophobic chromatography two peaks with activity were observed (Fig. 2A). As the second peak contained more than 90% of the activity recovered, this fraction was used for further chromatography on Mono S HR 5/5. A major contaminant could be removed from two distinct phytases, called P1 (elution time 37.6 min) and P2 (elution time 40.5 min) (Fig. 2B). A 16,500-fold purification of both enzymes was achieved with a recovery of 7 and 18%, respectively. Both enzymes exhibit an activity of about 8000 U/mg.

In the purified enzymes, there is a low acid phosphatase activity measurable. This activity was not separable through the whole purification procedure. In all steps the purification parameters were parallel for both activities.

The properties presented are nearly identical for both phytases P1 and P2, except the behavior on Mono S HR 5/5.

Molecular Properties

The molecular mass and the homogeneity of the purified enzymes were estimated by SDS–PAGE and gel filtration. Polyacrylamide gel electrophoresis under denaturing and nondenaturing conditions revealed only one single protein band after staining the gels by Coomassie or silver nitrate. Isoelectric focusing gave one major band corresponding to a p*I* of about 6. These results indicate that the phytases could be regarded as homogenous.

On a 10% SDS gel the molecular masses of the phytases were estimated to be approximately 42 kDa. The same value (43 kDa) was calculated after gel filtration of the native enzymes on a calibrated Sephacryl S-200 column. Consequently, the phytases are monomeric proteins. Both enzymes showed typical absorbance spectra for proteins with maxima at 278 nm and minima at 262 nm. No absorbance was observed in the visible region.

The amino acid compositions of the phytases as shown in Table II are based on a molecular mass of 42 kDa for both enzymes. The p*I* of 6 leads to the assumption that most of the Asx- and Glx-residues are present as aspartic acid and glutamic acid residues in the proteins. Because of the limited amounts of the enzymes it was only possible to determine the amino-terminal sequence of the phytase P2. Edman degradation of P2 gave a mixture of two distinct sequences:

Ser–Glu–Pro–Glu–Leu–Lys–Leu–Glu–Ala–Val–Val

Gln–Ser–Glu–Pro–Glu–Leu–Lys–Leu–Glu–Ala–Val.

Obviously, these two sequences are due to different processing of a preprotein.

Searching for homology of the amino-terminal sequence of phytase P2 in the EMBL-data bank revealed an identity with the pH 2.5 acid phosphatase from *E. coli*. The structure of that acid phosphatase was conducted from an incomplete nucleotide sequence (24). The only difference in the amino-terminal sequence of the phytase P2 is the exchange of the second Ser for Ala.

Enzymatic Properties

pH Optimum and pH stability. Phytase assay was performed from pH 1.0 to 9.0 using a variety of buffers

TABLE I

Purification Scheme

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	390	5811	2804	0.5	1	—
$(NH_4)_2SO_4$ precipitation	130	1820	2665	1.5	3	95
CM-Sepharose CL 6B	145	97.2	2019	20.8	43	72
DEAE-Sepharose CL 6B	85	20.4	1625	79.7	166	58
Phenyl-Sepharose CL 4B	440	1.76	841	473	985	30
Mono S HR 5/5 P1	2.0	0.024	192	8000	16660	7
Mono S HR 5/5 P2	1.2	0.063	505	8016	16700	18

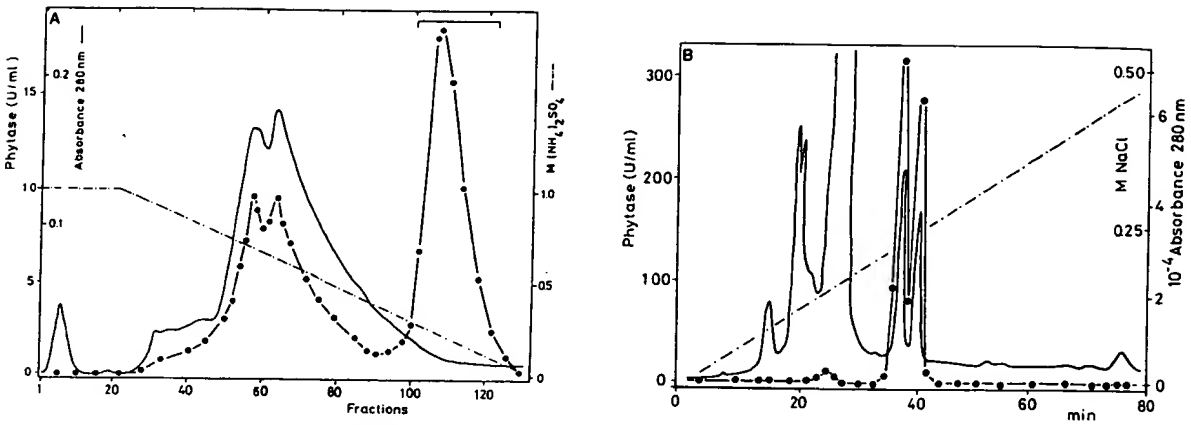


FIG. 2. Hydrophobic chromatography of the phytase active fraction obtained from DEAE–Sepharose CL 6B (A) and ion-exchange chromatography of fraction II from phenyl-Sepharose CL 4B on Mono S HR 5/5 (B). (●) phytase, (—) optical density at 280 nm, (-·-·), salt gradient.

(Fig. 3). The phytases have a single pH optimum at pH 4.5 and are virtually inactive above pH 7.0. The effect of the pH-value on enzyme stability was tested in the pH-range 1.0 to 9.0 at 4°C. Within 14 days the phytases do not lose any activity at pH levels ranging from 3.0 to 9.0. But at pH values < 3 the stabilities drop dramatically. At pH 2.0 40% and at pH 1.0 70% of the original activities are lost after 24 h.

Temperature optimum and thermal stability. The temperature profiles of the purified phytases were conducted from 10 to 85°C using the standard phytase assay at the given temperature. The temperature optimum was found to be 55°C and a sharp decrease in activity was observed at 60°C. The activation energy for the hydrolysis of phytate was calculated to be 58.5 kJ/mol from the Arrhenius plot. In order to check thermal stability, the phytases were incubated at different temperatures, cooled to room temperature, and assayed using the standard phytase assay. During a period of 1.0 h no loss of activity was observed from 30 to 50°C, while at 60°C only 24% of the activity remained and at 70°C no activity was detectable.

Kinetic parameters. To study the enzyme–substrate affinity and substrate selectivity, the kinetic parameters of the hydrolysis of phytate and PNPP were determined

TABLE II

Amino Acid Compositions of the Phytases P1 and P2

Amino acid residue	Phytase P1	Phytase P2	pH 2.5 acid phosphatase
Asx	38.1 (38)	37.7 (38)	40
Thr	22.3 (22)	24.4 (24)	32
Ser	21.6 (22)	23.2 (23)	27
Glx	49.5 (50)	50.0 (50)	43
Pro	16.4 (16)	16.4 (16)	16
Gly	31.3 (31)	32.2 (32)	38
Ala	65.7 (66)	61.4 (61)	60
Cys	1.6 (2)	1.4 (1)	n.d.
Val	17.9 (18)[a]	16.5 (17)[a]	39
Met	2.9 (3)	2.1 (2)	(1)
Ile	26.1 (26)[a]	29.8 (30)[a]	15
Leu	9.0 (9)[a]	8.6 (9)[a]	48
Tyr	2.4 (2)	3.0 (3)	6
Phe	8.3 (8)	7.7 (8)	13
Lys	12.4 (12)	10.5 (11)	25
His	7.0 (7)	7.3 (7)	1
Arg	15.2 (15)	15.1 (15)	13
Trp	n.d.	n.d.	n.d.

Note. Data of the pH 2.5 phosphatase taken from Dassa *et al.* (1).
[a] Value taken at 96 h.

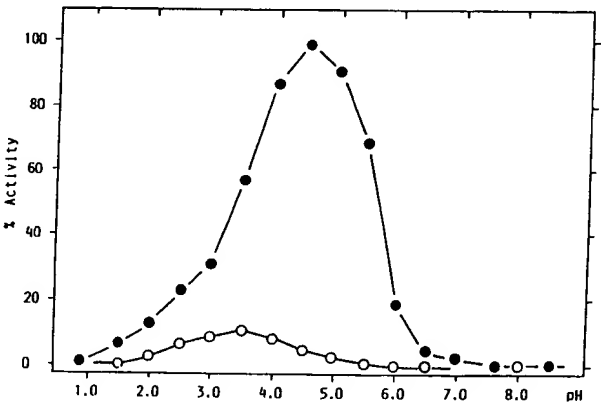


FIG. 3. Effect of pH on the activity of phytase P2. As substrate ( ) Na-phytate or (O) PNPP was used. The activity at pH 4.5 was taken as 100% when using Na-phytate as substrate. Buffers: pH 1–3.5, glycine/HCl; pH 3.5–6, Na-acetate/NaOH; pH 6–7 Tris/H-acetate; pH 7–9, Tris/HCl; pH 9–10, glycine/NaOH; each 0.1 M when using Na-phytate as a substrate. pH 1–6, Na-formiate/NaOH; pH 6–7, Tris/H-acetate; pH 7–9, Tris/HCl; pH 9–10 glycine/NaOH; each 0.25 M when using *p*-nitrophenyl phosphate as a substrate.

TABLE III

Kinetic Constants for the Hydrolysis of Na-phytate and PNPP by Phytase P2

Substrate	K_M (10^{-3} mol/liter)	k_{cat} (s^{-1})	k_{cat}/K_M (10^5 s^{-1} M^{-1})
PNPP	7.78	490	0.63
Phytic acid	0.13	6209	477.6

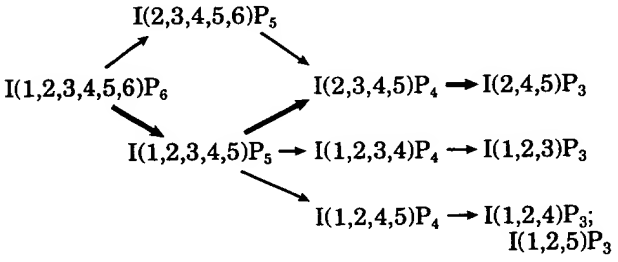
Note. Buffer, 0.1 M Na-acetate, pH 4.5; substrate concentration, 0.02–5 mM.

at pH 4.5. The results are summarized in Table III. The pronounced differences in the kinetic data for the hydrolysis of both substrates clearly indicate that the purified enzymes are phytases rather than nonspecific acid monophosphoesterases.

Like other phytases, the *E. coli* enzymes showed a substrate inhibition. The activities of the isolated phytases were inhibited at substrate concentrations > 1.5 mM.

Substrate selectivity. The action of the purified phytases in 0.1 M sodium acetate, pH 4.5, on several phosphate esters was tested. The relative rates of hydrolysis are summarized in Table IV. Phytate has the highest turnover number and the other phosphorylated compounds do not reach 10% of the phytate hydrolysis rate.

Hydrolysis pathway. In order to examine the action of phytase P2 on Na-phytate, the hydrolysis products were separated by HPLC ion pair chromatography and ion-exchange chromatography. Phytate is hydrolyzed in a stepwise manner. IP_6 is rapidly degradated and IP_5 is a short-lived intermediate. IP_4 is accumulated during the process and later is slowly hydrolyzed to IP_3 (Fig. 4). The remaining derivatives are further degradated to free *myo*-inositol. However, the hydrolysis rate is markedly decreased probably due to product inhibition (inorganic phosphate). From the analysis of the individual stereoisomers and of their amounts the following pathway was deduced. The major pathway is indicated by the bold arrows.



Effect of cations on enzyme activity. The study of the effect of metal ions on the enzyme activity reveals that none of them had an activating effect when used in concentrations between 10^{-4} and 10^{-3} M. Mg^{2+} and Hg^{2+} had

TABLE IV

Substrate Specificity

Substrate	Activity P1 (%)	Activity P2 (%)
Phytate	100	100
p-Nitrophenyl-phosphate	12.3	9.8
1-Naphthyl-phosphate	0.7	0.8
2-Naphthyl-phosphate	2.7	2.5
2-Glycerophosphate	1.9	1.7
Fructose 1,6-diphosphate	8.5	8.5
Fructose 6-phosphate	1.3	1.7
Glucose 6-phosphate	0.4	0.8
AMP	0	0
ADP	0	0
ATP	0	0
NADP	0	0
Na_2H_2-pyrophosphate	0	0
Pyridoxalphosphate	0	0
Phosphoserine	0	0
GTP	0	0

Note. Hydrolysis rate of Na-phytate was taken as 100%; 0.05 U of phytase was used.

no significant effect, while Mn^{2+} and Ca^{2+} were slightly disactivating. Cu^{2+} and Zn^{2+} showed strong inhibitory effects. The reduced phytase activity in the presence of Fe^{2+} and Fe^{3+} is attributed to a lower phytate concentration because of the appearance of a Fe-phytate precipitate.

Inhibition studies. When compounds which tend to chelate metal ions such as *o*-phenanthroline, EDTA, ox-

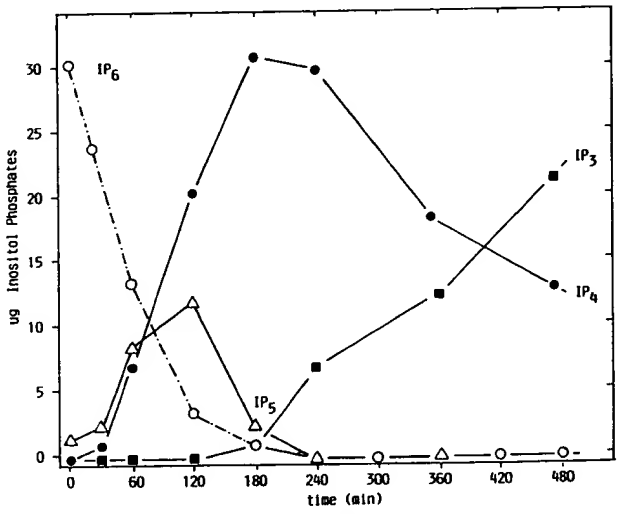


FIG. 4. Time course of the action of phytase P2 on Na-phytate. The enzymatic reaction products were separated by ion pair chromatography. IP_6–IP_3; *myo*-inositolhexakisphosphate to -trisphosphate.

alate, citrate, or tartrate were tested for their effect on enzyme activity, it was noticed that none of them was an inhibitor at concentrations from 10^{-4} to 10^{-3} M; citrate, in contrast, was slightly activating. Moreover, cyanide, azide, and arsenate did not show any effect. The absence of an effect of arsenate points to a missing participation of sulfhydryl groups in the active site of the enzyme. This suggestion was confirmed by testing the more specific sulfhydryl inhibitors 2-mercaptoethanol and iodoacetate, which did not show any effect either. The enzyme is also insensitive to the presence of the serine-specific reagent PMSF. The partial inactivation after incubation with EDC indicates that a carboxyl group takes part in the catalytic reaction. The strongest inhibitors were found in fluoride, molybdate, and vanadate. Fluoride, a known inhibitor of different phytases and phosphatases, inhibits the hydrolysis of phytate competitively with a K_i of 0.1 mmol/liter.

Comparison with the pH 2.5 acid phosphatase. The first hint of an identity of the phytase P2 and the pH 2.5 acid phosphatase was found during the investigation of the amino-terminal sequence, because there is only one difference in the first nine amino acid residues. To verify this suggestion some properties of the two enzymes were compared (Table V). Nearly all tested parameters of the phytase P2 are very similar to the pH 2.5 acid phosphatase. The difference in specific activity could be attributed to the usage of an FPLC-Mono S HR 5/5 column in order to remove the contaminants. This probably explains also the observed differences in the amino acid composition and in the maximal hydrolysis rate.

DISCUSSION

Two phytases were purified from *E. coli.* An ammonium sulfate precipitation and four column chromatographic steps led to two homogeneous protein preparations showing phytase activity. The enzyme preparation obtained after the hydrophobic chromatography appeared to be homogeneous during the SDS–PAGE. However, protein chemical studies indicated that this preparation must be contaminated by several other proteins. The phytase inactive contaminations could be effectively removed from the active proteins by chromatography on Mono S HR 5/5. Hereby two phytases were obtained, now designated phytases P1 and P2. Both preparations have nearly identical physicochemical and kinetic parameters.

The purified *E. coli* phytases share many kinetic parameters in common with other phytases (8, 9). They have an acidic pH optimum (pH 4.5) with a rapid drop in activity at pH-values above 6. In microorganisms a second optimum (pH 2.0–2.5) could often be observed, especially in aspergilli, but not in *E. coli.* A further characteristic of many phytases is their stability at high temperatures. *E. coli* phytases have a temperature optimum at 55°C and are denaturated at temperatures above 60°C. The activation energy of 58.5 kJ/mol can only represent some average value for the initial stage of hydrolysis.

Studies of some potent inhibitors point to a missing participation of cysteine and serine in the active site of the enzyme. The partial inactivation after incubation with EDC indicates that a carboxyl group takes part in the catalytic reaction.

TABLE V

Comparison of the Phytase P2 and the pH 2.5 Acid Phosphatase

	Phytase P2	pH 2.5 phosphatase
Amino-terminal sequence	Gln-Glu-Pro-Glu-Leu-Lys-Leu-Glu-Ala-Val-Val	Gln-Glu-Pro-Glu-Leu-Lys-Leu-Glu-Ser-Val-Val
Localization	Periplasma	Periplasma
Activity during growth	Highest activity under anaerobic conditions, during the exponential growth no activity observed	Highest activity under anaerobic conditions, during the exponential growth no activity observed
Specific activity	750 U/mg	326 U/mg
Molecular mass	42 kDa	45 kDa
Isoelectric point	6.0 ± 0.4	6.3
Stability in 1 M formic acid	Soluble and stable for ca. 4 h	Soluble and stable for ca. 2 h
pH optimum, PNPP as substrate	In glycine pH 2.5, in formiate pH 3.5, activity ratio for formiate:glycine at pH 3.5 = 0.45	In glycine pH 2.5, in formiate pH 3.3, activity ratio for formiate:glycine at pH 3.5 = 0.5
Substrate specificity	PNPP 100%, fructose 1,6-diphosphate 34%, GTP 46%	PNPP 100%, fructose 1,6-diphosphate 37%, GTP 45%
Kinetic constant	K_M = 2.64 μM V_{max} = 703 μmol min^{-1} mg^{-1}	K_M = 2.7 μM, V_{max} = 208 μmol min^{-1} mg^{-1}
Inhibitors	fluoride: K_I = 82 μM (competitive), phosphate: K_I = 11.9 mM, tartrate: K_I = 730 μM (competitive)	fluoride: K_I = 60 μM (competitive), phosphate: K_I = 13 mM, tartrate: K_I = 700 μM (competitive)

Note. The data of the pH 2.5 acid phosphatase were taken from Dassa *et al.* (1).

Phytase is considered to be a special type of acid phosphatase, which is capable of splitting off phosphate from phytate as well as other diversified organophosphates. The *E. coli* phytases have a high specificity to phytate and they are fast-acting enzymes with a turnover rate of approximately 6200 per second. The only data available on molecular activities of other phytases are 220 per second for *Aspergillus ficuum* NRRL 3135 phytase (8) and less than 10 per second for soybean phytase (25).

Phytases from microorganisms are considered as 3-phytases. However, the phytase P2 is obviously a 6-phytase as the phosphoester bond at position 6 of the *myo*-inositol residue is preferentially hydrolyzed. The rate of hydrolysis at position 3 is only 1/7 of that at position 6. The *E. coli* phytases are capable of degrading phytate to free *myo*-inositol, but the later steps were inhibited by the released phosphate.

Searching for homology of the amino-terminal sequence of phytase P2 in the EMBL-data bank revealed an identity with the pH 2.5 acid phosphatase from *E. coli*. The structure of that acid phosphatase was conducted from an incomplete nucleotide sequence (24). The organization of the gene and the whole structure of this protein is not yet established. The kinetic parameters and molecular mass of our purified enzyme(s) correspond very well to that of Dassa *et al.* (1) when testing phytase P2 with PNPP at pH 2.5. In contrast to Dassa *et al.* (1), we achieved a threefold higher purification with reference to the specific activity. This could be attributed to the usage of an FPLC Mono S HR 5/5 column in order to remove the contaminants. This probably explains the observed differences in the amino acid composition of the phytases and the pH 2.5 acid phosphatase. If both enzymes are really identical, the pH 2.5 acid phosphatase should better be regarded as a phytase. Further work is in progress to elucidate the structure of the phytase P2/P1 and to overexpress the enzymes.

ACKNOWLEDGMENTS

We are indebted to Professor Mayr, University of Bochum, for performing the analysis of the stereoisomers of the individual *myo*-inositol phosphate derivatives. The introduction to the method of Sandberg by Dr. Müller, Federal Research Centre for Nutrition Karlsruhe, is greatly acknowledged.

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HD&P Docket No. 6550-000038USB MSU ID number 99-005F ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: J. Frost and C. Hansen

Application No.: 09/937,243
Filed: September 21, 2001

Group No.: 1652
Examiner: David J. Steadman

For: SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1.2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1 PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDROGENASE

Commissioner for Patents
Washington, D.C. 20231

STATEMENT OF BIOLOGICAL CULTURE DEPOSIT

I, PAUL M. HURST, the Assoc VP Research & Graduate Studies of Board of Trustees Operating Michigan State University, Assignee of the above-referenced patent application as recorded in the United States Patent and Trademark Office on May 10, 2002 by reel and frame number 012886/0937, hereby state:

1. That the following cultures referred to in the specification of this application have been deposited:

Strain:	Accession number:
E. coli JWF1/pAD1.88A	207153
E. coli JWF1/pAD2.28A	207154

2. That the date of the above deposit is before the U.S. filing date of this application.

3. That the name and address of the depository is:

American Type Culture Collection
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Manassas, VA 20110-2209

4. That a statement that the cultures deposited with the above named depository was viable and was capable of reproduction, if appropriate, on the date of deposit is attached. Such statement was executed by the Administrator of the Patent Depository.

5. That, with respect to the permanence of the cultures deposited, the depository is an official depository, in accordance with the Budapest Treaty for the above deposited cultures.

6. That should the microorganism(s) mutate, become nonviable or be inadvertently destroyed, applicants will replace such microorganism(s) for at least 30 years from the date f the original deposit, or at least 5

years from the date of the most recent request for release of a sample or for the life of any patent issued on the above-mentioned application, whichever period is longer.

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Michigan State University
Attn: Amy Dean
Department of Chemistry
E. Lansing, MI 48824-1322

Deposited on Behalf of: Michigan State University

Identification Reference by Depositor:	**ATCC Designation**
Escherichia coli JWF1/pAD1.88A	207153
E. coli JWF1/pAD2.28A	207154

The deposits were accompanied by: ___ a scientific description X a proposed taxonomic description indicated above. The deposits were received March 10, 1999 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested March 17, 1999. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository **Date: March 17, 1999**

cc: DeAnn F. Smith

Amendment and Response to Office Action
Application No.: 09/937,243
Atty. Docket No.: 6550-000038/USB
Attachment 6